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E. coli MUTANTS DEFECTIVE IN THE CONVERSION OF pppGpp TO ppGpp

by

© Christopher R. Somerville

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *E. coli* mutants defective in the conversion of pppGpp to ppGpp, submitted by Christopher R. Somerville, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

Mutants of *E. coli* which accumulate abnormally high levels of pppGpp (guanosine 5'-triphosphate-3'-diphosphate) during amino acid or carbon source starvation have been isolated. The mutation responsible for this effect has been mapped to a previously undescribed locus designated as *gpp*. These mutants are defective in a nuclease which appears to specifically hydrolyze pppGpp to ppGpp. Chromatographic separation of the various pppGpp*ase* activities in the wild type strain and the *gpp* mutants revealed that the mutants are missing two of the five separable pppGpp*ases*. For this reason, it is suggested that the *gpp* enzyme may normally function in several aggregated forms.

Following the onset of amino acid starvation of the *gpp* mutants, pppGpp accumulates more rapidly than ppGpp. This effect, which is ascribed to a reduction in the rate of hydrolysis of pppGpp to ppGpp, substantiates the proposal that pppGpp is a major precursor of ppGpp.

In contrast to the wild type, *gpp* mutants accumulate pppGpp during carbon source downshift. This observation, which indicates that substantial (p)ppGpp synthesis occurs during downshift, is inconsistent with previous models in which the downshift-induced accumulation of ppGpp was attributed to reduced turnover of ppGpp.

The *spoT* gene appears to be involved in ppGpp degradation. It was, therefore, expected that a *gpp spoT* double mutant would be partially defective in both ppGpp and pppGpp degradation. There is no apparent reason to expect that such a combination would be inviable. However, several lines of evidence suggest that such a combination is lethal. The isolation of a putative *spoT*^{ts} mutant permitted the con-

struction of a *gpp spoT*^{ts} strain. The *spoT*^{ts} mutant is unusual in that it appears to have a normal rate of ppGpp degradation, but is typical in that, following amino acid starvation at 43.5°, pppGpp does not accumulate. As expected, the *gpp spoT*^{ts} mutant accumulates both ppGpp and pppGpp. However this mutant does not accumulate RNA at 46°. Since this effect could not be attributed to the levels of (p)ppGpp, it is concluded that *spoT* and *gpp* might be involved in some process in addition to the degradation of ppGpp and pppGpp.

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LIST OF ABBREVIATIONS

A_{450} unit	That concentration of cells which yeilds an optical density of 1.0 at 450 nm
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
cAMP	Cyclic adenosine 3',5'-monophosphate
CPM	Counts per minute
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EF-G	Elongation factor G
EF-T	Elongation factor T
GDP	Guanosine 5'-diphosphate
GTP	Guanosine 5'-triphosphate
KOAc	Potassium acetate
LiCl	Lithium chloride
$Mg(OAc)_2$	Magnesium acetate
MS-I	Magic spot I (ppGpp)
MS-II	Magic spot II (pppGpp)
NH_4OAc	Ammonium acetate
PEI	Poly(ethylene)imine
PEP	Phosphoenolpyruvate
ppGpp	Guanosine 5'-diphosphate-3'-diphosphate
pppGpp	Guanosine 5'-triphosphate-3'-diphosphate
(p)ppGpp	ppGpp and pppGpp
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
Tris-Cl	Chloride salt of Tris(hydroxymethyl)aminomethane
Tris-OAc	Acetate salt of Tris(hydroxymethyl)aminomethane

INTRODUCTION

The relA gene

In many strains of *Escherichia coli*, stable RNA synthesis is abruptly curtailed upon amino acid starvation. Stent and Brenner (1961) termed this phenomenon the "stringent response", and demonstrated that a mutation at a single locus could abolish the effect. A spontaneous mutation, which they designated as RC^{rel}, caused the relaxation of RNA synthesis in that RNA accumulated following amino acid starvation. Additional mutants at this locus, subsequently designated as *relA* (Bachmann *et al.*, 1976), were isolated by Fiil and Friesen (1968). The observation that *relA* mutants are recessive in merodiploids (Fiil, 1969) suggests that the *relA* gene product is a cytoplasmic component.

The first insight into the mechanism governing the stringent response was provided by Fangman and Neidhardt (1964), who demonstrated that, in a $relA^+$ strain, inactivation of an aminoacyl-tRNA synthetase resulted in the stringent response. This result, which was obtained even in the presence of a full complement of amino acids, indicated that stringency is a response to depletion of at least one of the aminoacyl-tRNA pools, rather than to the depletion of the amino acid pool *per se*. Cashel and Gallant (1969) and Cashel (1969) subsequently reported that, in $relA^+$ strains, amino acid starvation or inactivation of an aminoacyl-tRNA synthetase resulted in the rapid accumulation of two highly phosphorylated nucleotides, originally designated MSI and MSII (Magic Spot I & II). They proposed that a reaction

normally involved in protein synthesis idles during the stringent response and produces the MS-nucleotides. They also proposed that the accumulation of MS-nucleotides could be a causative factor in the cessation of RNA synthesis and the other characteristics of the stringent response. The identities of MSI and MSII have been established as guanosine 3'-diphosphate-5'-diphosphate (ppGpp) and guanosine 3'-diphosphate-5'-triphosphate (pppGpp) (Cashel and Kalbacher, 1970; Sy and Lipmann, 1973).

The *in vitro* synthesis of the MS-nucleotides was elegantly demonstrated by Haseltine *et al.*(1972) and Haseltine and Block (1973). They showed that ppGpp and pppGpp could be synthesized from ATP and GTP in response to the non-enzymatic binding of an uncharged tRNA to the acceptor site on the 70S ribosome-mRNA complex. They also demonstrated that a factor (stringent factor), which is present in the 0.5 M NH_4Cl ribosomal wash from *relA*$^+$ but not *relA*$^-$ cells, is necessary for the reaction to occur. They concluded that the stringent factor is either the product of the *relA* gene, or a protein modified by the action of the *relA* gene. Sy *et al.* (1973) and Block and Haseltine (1975) have extended this approach by demonstrating that (p)ppGpp synthesis can proceed in the absence of ribosomes, mRNA, and tRNA, when purified stringent factor is incubated in a simple mixture of buffer, salts and substrates. The coidentity of the stringent factor and the product of the *relA* gene has also been inferred from the differential rates of thermal inactivation of stringent factor activity from leaky *relA* mutants (Block and Haseltine, 1973). The *in vitro* synthesis of (p)ppGpp has been the subject of several reviews (Block and Haseltine, 1974; Lipmann and Sy, 1976).

The stringent response

Until recently, the stringent response was interpreted as being primarily a regulatory mechanism for adjusting the rate of synthesis of stable RNA. It now appears that many other cellular functions are under some form of stringent control. A partial list of additional activities which are under negative control includes lipid and phospholipid synthesis, nucleotide synthesis and transport, glycolysis, respiration and translation (Cashel and Gallant, 1974; Gallant and Lazzarini, 1976). The rate of transcription of some amino acid biosynthetic pathways appears to be stimulated by the action of the *relA* gene (Stephens *et al.*, 1975; Somerville and Ahmed, 1977), but the rate of transcription of an arginine biosynthetic gene appears to be negatively regulated (Gallant *et al.*, 1970; Yang *et al.*, 1974). The effects of the stringent response on the components of the transcription and translation apparatus cannot be generalized. The synthesis of some components appears to be negatively regulated, some positively regulated, and others do not appear to be under this form of regulatory control (Dennis and Nomura, 1975; Reeh *et al.*, 1976; Furano and Wittel, 1976).

The mechanism by which the *relA* gene mediates the regulation of such diverse components of the bacterial cell remains uncertain. The involvement of ppGpp as a regulatory signal can be inferred from *in vivo* results obtained during amino acid starvation. Accumulation of ppGpp is almost always accompanied by a concomitant decrease in stable RNA accumulation (Cashel and Gallant, 1974; Cashel, 1975; Gallant and Lazzarini, 1976; Fiil *et al.*, 1977). A single equivocal counter example has been reported (Gallant *et al.*, 1977), and challenged (Chaloner-Larsson and Yamazaki, 1977). The results of *in vitro* ex-

periments designed to test the effects of ppGpp as a modifier of transcription are generally consistent with *in vivo* results. Cashel (1970) originally presented evidence that ppGpp interacts with purified core RNA polymerase to modify the specificity of transcription. Positive and negative effects on the rate of *in vitro* transcription have subsequently been reported with a variety of templates and assay conditions (Yang *et al.*, 1973; Reiness *et al.*, 1975; Stephens *et al.*, 1975; Travers, 1976a; Van Ooyen *et al.*, 1976). These studies have generally led to the conclusion that ppGpp interacts directly with core RNA polymerase to alter transcriptional specificity. Aboud and Pastan (1975) have, however, reported the partial purification of a factor which may be required for the stimulation of *in vitro* RNA synthesis by ppGpp. Several other authors have not been able to demonstrate a specific effect of ppGpp on *in vitro* transcription (Haseltine, 1972; Murooka and Lazzarini, 1973). A possible explanation for these discrepancies is implicit in the well known sensitivity of *in vitro* transcription assays to slight variations in the experimental conditions (Travers, 1976b).

The downshift response

When a growing culture of *E. coli* is shifted from a medium containing a good carbon source to a medium containing a poor carbon source, the cells undergo a rapid transition in cellular activity (Maaløe and Kjeldgaard, 1966). One of the changes is the cessation of stable RNA synthesis and accumulation. Although the regulatory mechanism governing this response is obscure, ppGpp may be involved

as a regulatory signal. This is inferred from the observation that ppGpp accumulates within minutes of downshift in certain *relA*$^+$ strains, but shows a substantially reduced rate of accumulation in otherwise isogenic *relA*$^-$ strains (Lazzarini *et al.*, 1971; Winslow, 1971; Hansen *et al.*, 1975). Although *relA*$^-$ strains also restrict the rate of stable RNA synthesis, they exhibit a significantly longer lag phase before resuming growth on the alternate carbon source. This property of *relA*$^-$ strains has been used as an enrichment procedure in the isolation of new *relA* mutants (Fiil and Friesen, 1968).

The mechanism by which downshift provokes the accumulation of ppGpp is obscure. Analysis of the turnover rate for ppGpp, under these conditions, suggests that the increase in the level of ppGpp can be largely accounted for by a reduction in the rate of turnover, without any appreciable change in the rate of synthesis (Gallant *et al.*, 1972; Friesen *et al.*, 1975; De Boer *et al.*, 1976). Thus, the slow accumulation of ppGpp observed in *relA* mutants can be accounted for by the fact that all of the available *relA* mutants are leaky and maintain basal levels of ppGpp which are comparable to those observed in the wild type. The proposal is also consistent with the observation that cells undergoing downshift do not accumulate pppGpp, a feature which distinguishes the downshift response from the response obtained during amino acid starvation.

Several models have been proposed to account for the presumed reduction in ppGpp turnover during downshift. Laffler and Gallant (1974) and Stamminger and Lazzarini (1974) suggested that downshift could cause a reduction in the availability of a high energy phosphate donor, required for the conversion of ppGpp to pppGpp. An alternate

scheme is based on the assertion that pppGpp is a precursor of ppGpp, and that ppGpp is subsequently converted to an unidentified compound by removal of the 3'-pyrophosphate group (Chaloner-Larsson and Yamazaki, 1976; Fiil *et al.*, 1977; Kari *et al.*, 1977). According to this scheme, downshift occasions a depletion of a phosphate (or pyrophosphate) acceptor, resulting in the concomitant reduction in the rate of ppGpp degradation. Thus, both models propose that the turnover rate of ppGpp is regulated during downshift by substrate limitation. The recent demonstration of *in vitro* degradation of ppGpp (Sy, 1977; Heinemeyer and Richter, 1977) should facilitate the resolution of this problem.

Whatever the precise mechanism for the downshift induced accumulation of ppGpp, it appears that the bacterial cell may utilize the same regulatory signal (ppGpp) to adjust macromolecular composition in response to substantially different forms of nutritional stress.

Braedt and Gallant (1977) have recently reported an unexpected influence of the *relA* gene upon the downshift induced accumulation of cyclic adenosine 3',5'-monophosphate (cAMP). They observed that, following carbon source downshift, a *relA* mutant accumulates a two- to three-fold excess of cAMP over that observed in a *relA*$^+$ strain. Since the effect could not be attributed to the accumulation of ppGpp, they suggested that the *relA* gene product may have another unidentified function, such as the formation of a regulatory nucleotide other than ppGpp.

Mutations affecting ppGpp and pppGpp accumulation

In addition to the *relA* gene, a number of other loci have been identified which affect MS-nucleotide metabolism (reviewed by Cashel, 1975). A mutant designated as *relB* is characterized by the delayed onset of relaxed control of RNA accumulation following amino acid starvation (Lavalle, 1965; Diderichsen *et al.*, 1977). Although little has been published regarding this mutant, the phenotype is reportedly due to the accumulation of a substance which inhibits protein synthesis and (p)ppGpp accumulation. Another class of mutants with a relaxed phenotype, which does not accumulate ppGpp during amino acid starvation, has been isolated (Friesen *et al.*, 1974). These mutants, originally designated as *relC*, have been characterized as having an altered L11 ribosomal protein (Parker *et al.*, 1976), and are now designated as *rplK* (Bachmann *et al.*, 1976). Presumably, the L11 protein participates in the synthesis of the MS-nucleotides by the *relA* protein, perhaps by facilitating the binding of uncharged tRNA or stringent factor to the ribosome.

Laffler and Gallant (1974) have mapped a spontaneous mutation designated as *spoT*. This mutant accumulates excessive amounts of ppGpp during amino acid starvation without the concomitant accumulation of significant amounts of pppGpp. The observation that *spoT* mutants exhibit the stringent response without accumulating pppGpp has served to focus attention on ppGpp as the regulatory signal for the stringent response. A careful examination of the kinetics of residual pppGpp synthesis in these mutants revealed that, following amino acid starvation, *spoT* mutants synthesize a quick burst of pppGpp which

does not approach wild type levels and rapidly declines to a barely detectable level (Chaloner-Larsson and Yamazaki, 1976; Fiil *et al.*, 1977). This effect has been interpreted as evidence that *spoT* mutants are competent to synthesize pppGpp, but that the synthesis is inhibited, possibly by the high level accumulation of ppGpp.

The most informative characteristic of the *spoT* mutants is that, following the release of starvation conditions, the rate of ppGpp decay is decreased as much as twenty-fold (Laffler and Gallant, 1974; Stamminger and Lazzarini, 1974; Fiil *et al.*, 1977). This aspect of the *spoT* phenotype has been interpreted as evidence that the *spoT* gene product is required for the enzymatic degradation of ppGpp. This interpretation is substantiated by the recent demonstration that the ribosomal wash from a *spoT* mutant is partially defective in the *in vitro* degradation of ppGpp (Sy, 1977).

Several other mutants have been reported which modify the stringent phenotype (see review by Cashel, 1975). These mutants are not relevant in the present context since they do not alter the pattern of (p)ppGpp accumulation, or have not been characterized in this respect. An abbreviated linkage map of *E. coli*, illustrating the positions of the various loci involved in (p)ppGpp metabolism, is presented in Figure 1.

The pathway of ppGpp synthesis

Although the general mechanisms responsible for the synthesis and degradation of the MS-nucleotides have been elucidated, many of the details remain obscure. A fundamental problem has been the inability to demonstrate a direct precusor-product relationship

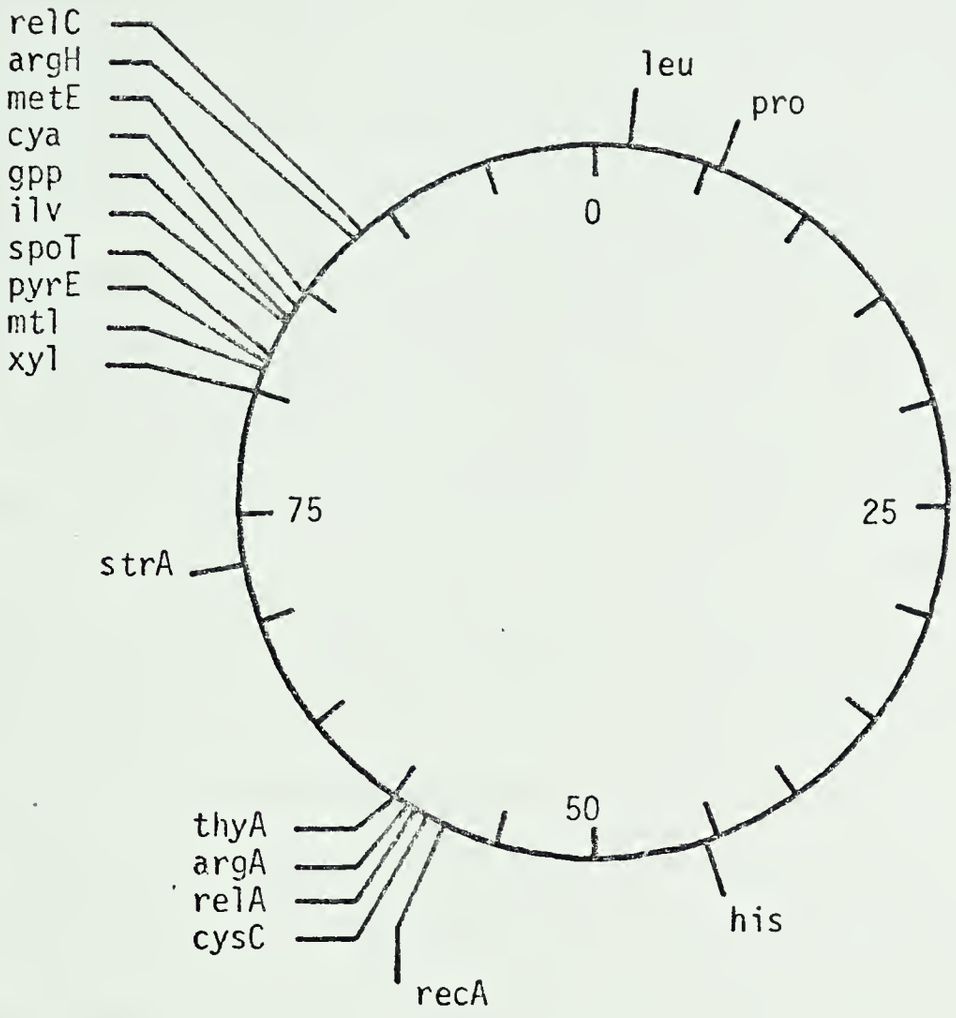

Figure 1 - Linkage map of *E. coli* K12 illustrating the position of some of the genes relevant to this study. The map location of all loci except *gpp* is as described by Bachmann *et al.* (1976).

between the two MS-nucleotides. The *in vitro* reaction has not been particularly useful in this respect since GDP and GTP are virtually equivalent acceptors of the pyrophosphate group from ATP (Sy, 1974). The fifty-fold excess of GTP over GDP in the intact cell makes it unlikely that ppGpp and pppGpp are synthesized independently (Fiil *et al.*, 1977). It must be noted however that, *in vivo*, the substrate for MS-nucleotide synthesis may not be obtained directly from the free-nucleotide pool, but may be bound to the ribosome by one of the translation factors.

The *spoT* mutant, which is clearly defective in the degradation of ppGpp and simultaneously defective in pppGpp accumulation, was initially interpreted as evidence that ppGpp is the precursor of pppGpp (Laffler and Gallant, 1974; Stamminger and Lazzarini, 1974; Cashel and Gallant, 1974; De Boer *et al.*, 1976). Such a scheme was particularly appealing since it suggested that a phosphorylation step was required for the conversion. Thus, the accumulation of ppGpp during downshift could be accounted for by the depletion of a high energy phosphate donor derived either directly or indirectly from the pathways of carbon catabolism. A major difficulty with this model is the transient (albeit low level) accumulation of pppGpp observed in *spoT* strains following amino acid starvation (Chaloner-Larsson and Yamazaki, 1976; Kari *et al.*, 1977; Fiil *et al.*, 1977). Also, Fiil *et al.* (1977) examined the kinetics of ^{3}H-guanosine entry into GTP, ppGpp, and pppGpp following amino acid starvation, and observed that the specific activity of pppGpp increases more quickly than that of ppGpp. Therefore, they concluded that pppGpp is the precursor of ppGpp. Similar results in several other laboratories support such a scheme (Chaloner-Larsson and Yamazaki, 1976; Kari *et al.*, 1977; De Boer *et al.*, 1977). The general

model proposed by these authors is that pppGpp is synthesized from ATP and GTP by the action of the *relA* gene product, and is subsequently converted to ppGpp by one or more unidentified enzymes. The *spoT* gene product catalyzes the conversion of ppGpp to an unidentified compound without the concomitant accumulation of pyrophosphate (Kari *et al.*, 1977). The downshift induced accumulation of ppGpp might, therefore, be accounted for by the depletion of a suitable pyrophosphate acceptor. To account for all of the observations, it appears necessary to postulate that, in *spoT* mutants, the accumulation of ppGpp results in feedback inhibition of pppGpp synthesis. This could explain the transient burst of pppGpp synthesis observed at the onset of amino acid starvation, and the reduced rate of ppGpp *synthesis* under these conditions (Fiil *et al.*, 1977). The fact that high level ppGpp accumulation in $spoT^+$ cells does not result in a comparable reduction of pppGpp synthesis, suggests that the *spoT* gene product could be directly involved in pppGpp synthesis, perhaps in a regulatory capacity.

Since both of the foregoing models are largely unsubstantiated by genetic evidence, it was considered worthwhile to attempt to define the precusor-product relationship of the MS-nucleotides by the selection of additional mutants. The correct assignment of such relationships would be particularly useful in defining the nature of the events which result in downshift induced accumulation of ppGpp. A particularly informative mutant was considered to be one in which the hydrolysis of pppGpp was blocked. If pppGpp is the precusor of ppGpp, such a mutant would be expected to accumulate elevated levels of pppGpp during amino acid starvation, but would not accumulate ppGpp.

The feasibility of finding such a mutant could not be assessed in advance. Elongation factors G (EF-G) and T (EF-T) are capable of the ribosome-dependent hydrolysis of pppGpp to ppGpp *in vitro* (Hamel and Cashel, 1973; Cochran and Byrne, 1974). However, since the *in vivo* significance of this reaction had not been established, it was considered possible that other dispensable enzymes might perform this function *in vivo*. A search was, therefore, carried out for mutants which accumulated excessive amounts of pppGpp during amino acid starvation. Several such mutants were recovered, mapped, and characterized. These mutants appear to be totally defective in a dispensable nuclease which hydrolyzes pppGpp to ppGpp. However, these mutants accumulate ppGpp at approximately the same level as wild type strains. Evidence is presented which suggests that this is due to the presence of several other enzymes which are capable of pppGpp hydrolysis. Although it has not been possible to isolate a mutant which is totally defective in pppGpp hydrolysis, the results of several experiments, reported here, support a scheme in which pppGpp is a major precursor of ppGpp.

MATERIALS AND METHODS

Bacterial and bacteriophage strains

The genotypes and sources of the bacterial strains used in this study, all of which are derived from *E. coli* K12, are described in Table 1. The generalized transducing phage P1*vir* was obtained from Dr. A. Ahmed's collection. The male specific phage R17 was obtained from Dr. W. Paranchych.

Media

The medium of Davis and Mingioli (as described by Roth, 1970), containing 0.2% of the appropriate carbon source, was generally used as the minimal medium. The Tris buffered minimal medium of Kaempfer and Magasanik (1967), containing KH_2PO_4 at a final concentration of 0.4 mM, was used in those instances where cells were labelled with $^{32}PO_4$. These media were supplemented, when necessary, with 20 ug/ml of the required L-amino acid, 10 ug/ml thiamine, and nucleosides at 50 ug/ml. L-broth or L-agar was generally used as the complete medium (Miller, 1972).

Genetic techniques

Transductions were performed with the generalized transducing phage P1*vir* according to the procedure described by Lennox and Yanofsky (1959). The methods for mutagenesis, Hfr matings, and F' plasmid transfers are described in Miller (1972).

Table 1 - Bacterial strains

Strain	Genotype	Source / Derivation
AB1206	F14 *ilv-argH / Δ(ilv-argH) proA2 lacY1 galK2 supE44 his-4 str-8 thi-1 tfr-3*	B. Bachmann
AB1450	F^- *lac gal his strA mal xyl ilv metB arg thi*	"
AB2547	Hfr $supN_{ochre}$ *xyl-4 ilvD188 arg-1 pur-1*	D. Mount
AT2243	Hfr C *tonA22 relA1 pyrE41 spoT*	B. Bachmann
KL16-99	Hfr *drm-3 recA1 thi-1*	"
KL209	Hfr *supE44 malB16*	"
KL266	F^- *leu-6 ara-14 lacZ36 proC32 hisF860 cysC43 thyA54 str-109 spc-15 xyl-5 mtl-1 metE70 thi-1*	"
LS853	F^- *trpR55 trpA9605 his-85 Δcya-2*	"
R1	Hfr *relA1 metB1*	"
Ra-2	Hfr *sfa-4 mal-28*	"
CS126	Hfr *relA1 argA52 thi-1*	A derivative of KL16
CS251	F^- $lacZ_{am}$ *metG146 metG155 hisC3 thyA strA25 xyl-5 mtl-1 argH rpoB glpK nalA*	A derivative of AB1111
CS305	Hfr *thi*	A derivative of KL16
CS401	F^- *strA mtl ilv glpK metB argH malA thi* sup^+_{am}	A derivative of AB1450

Table 1 - continued

Strain	Genotype	Source / Derivation
CS403	F^- *strA mtl ilv glpK metB argH malA thi* sup^+_{am} *gpp-1*	By transduction, P1(MS240) x CS401, ilv^+ selected
CS408	F^- *hisF860 recA1 str-109 spc-15 xyl-4 or 5 gpp-1 metE70 thi*	By conjugation, KL16-99 x CS416, thy^+ str^r selected
CS409	F^- *hisF860 recA1 str-109 spc-15 xyl-4 or 5 gpp-1 metE70 thi* km^r	A spontaneous Kanamycin resistant mutant of CS408
CS410	F^- *hisF860 cysC43 thyA54 str-109 spc-15 xyl-4 or 5 ilvD188 metE70 thi*	By conjugation, AB2547 x KL266, mtl^+ str^r selected
CS411	F^- *leu-6 ara-14 proC32 lacZ36 hisF860 cysC43 str-109 spc-15 pyrE41 metE70 thi thyA54*	By conjugation, AT2243 x KL266, xyl^+ str^r selected
CS412	F^- *leu-6 ara-14 hisF860 cysC43 thyA54 str-109 spc-15 pyrE41 spoT metE70 thi*	By conjugation, AT2243 x KL266, xyl^+ str^r selected
CS414	F^- *hisF860 cysC43 thyA54 str-109 spc-15 xyl-4 or 5 Δcya-2 metE70 thi*	By transduction, P1(LS853) x CS410, ilv^+ selected

Table 1 - continued

Strain	Genotype	Source / Derivation
CS415	F⁻ *hisF860 cysC43 thyA54 str-109 spc-15 xyl-4 or 5 ilvD188 gpp-1 thi*	By transduction, P1(CS403) x CS410, met$^+$ selected
CS416	F⁻ *hisF860 cysC43 thyA54 str-109 spc-15 xyl-4 or 5 metE70 gpp-1 thi*	By transduction, P1(CS403) x CS410, ilv$^+$ selected
CS417	F⁻ *hisF860 cysC43 thyA54 str-109 spc-15 xyl-4 or 5 metE70 thi*	By transduction, P1(CS403) x CS410, ilv$^+$ selected
CS418	F⁻ *hisF860 cysC43 thyA54 str-109 spc-15 xyl-4 or 5 ilvD188 Δcya-2 thi*	By transduction, P1(LS853) x CS410, met$^+$ selected
CS419	F⁻ *leu-6 ara-14 proC32 lacZ36 hisF860 cysC43 thyA54 str-109 spc-15 pyrE41 gpp-1 thi thyA54*	By transduction, P1(CS403) x CS411, met$^+$ selected
CS421	F⁻ *relA1 argA52 str-109 spc-15 xyl-4 or 5 gpp-1 metE70 thi ilv*	By conjugation, CS126 x CS416, thy$^+$ str^r selected
CS422	F⁻ *str-109 spc-15 xyl-4 or 5 gpp-1 metE70 thi ilv*	By transduction, P1(CS305) x CS421, arg$^+$ selected

Table 1 - continued

Strain	Genotype	Source / Derivation
CS423	F⁻ *str-109 spc-15 xyl-4 or 5 thi ilv*	By transduction, P1(CS305) x CS422, met$^+$ selected
CS424	F⁻ *relA1 argA52 str-109 spc-15 xyl-4 or 5 thi ilv*	By transduction, P1(CS305) x CS421, met$^+$ selected
CS425	F⁻ *leu-6 ara-14 lacZ36 proC32 hisF860 cysC43 thyA54 str-109 spc-15 xyl-5 mtl-1 gpp-2 thi-1*	By transduction, P1(MS556) x KL266, met$^+$ selected
CS426	" " " " *gpp-3* " " "	" P1(MS551)
CS427	" " " " *gpp-4* " " "	" P1(MS631)
CS428	F⁻ *leu-6 ara-14 lacZ36 proC32 hisF860 cysC43 thyA54 str-109 spc-15 xyl-5 mtl-1 gpp-5 thi*	By transduction, P1(MS552) x KL266, met$^+$ selected
CS429	" " " " *gpp-6* " " "	" P1(MS641)
CS430	" " " " *gpp-7* " " "	" P1(MS573)

Table 1 - continued

Strain	Genotype	Source / Derivation
CS431	" " " " *gpp-8* " " "	" P1(MS217)
CS432	" " " " *gpp-9* " " "	" P1(MS50)
CS444	F⁻ *leu-6 ara-14 proC32 lacZ36 hisF860 cysC43 str-109 spc-15 spoT*^{ts} *metE70 thi thyA54*	By transduction, P1(MS192) x CS411, pyrE^{+} selected
CS445	F⁻ *leu-6 ara-14 proC32 lacZ36 hisF860 cysC43 str-109 spc-15 spoT*^{ts} *gpp-1 thi thyA54*	By transduction, P1(CS403) x CS444, metE^{+} selected
MS50	F⁻ *lacZ*$_{am}$ *metG146 metG155 hisC3 thyA strA25 xyl-5 mtl-1 gpp-9 argH rpoB glpK nalA*	NG mutagenesis of CS251
MS192	" *spoT*^{ts} " " " " " "	"
MS217	" *gpp-8* " " " " " "	"
MS240	" *gpp-1* " " " " " "	"
MS551	F⁻ *strA mtl gpp-3 glpK metB argH malA thi sup*$^{+}_{am}$	NG mutagenesis of CS401
MS556	" " *gpp-2* " " " " "	"
MS552	" " *gpp-5* " " " " "	"

Table 1 - continued

Strain	Genotype	Source / Derivation
MS573	F⁻ *strA mtl gpp-7 glpK metB argH malA thi* sup^+_{am}	NG mutagenesis of CS401
MS631	" " *gpp-4* " " " " "	"
MS641	" " *gpp-6* " " " " "	"
W2	F⁻ *spoT thr leu pro gal his arg thi*	J. Gallant

Scoring the rel phenotype

The rel phenotype was scored in liquid cultures by measuring the rate of ^{3}H-uracil incorporation into acid precipitable material (Stent and Brenner, 1961). At time 0, the culture was supplemented with ^{3}H-uracil (250 uCi/umole; 0.04 umole/ml) and 100 ug/ml cytidine to reduce incorporation of label into DNA. Amino acid starvation was conveniently imposed on cultures of val^{S} (ilv^{+}) strains by the addition of L-valine to a final concentration of 600 ug/ml. Under these conditions, the exogenous valine causes repression of isoleucine synthesis, resulting in effective starvation for isoleucine (Leavitt and Umbarger, 1962). The starvation was alleviated, when necessary, by the addition of L-isoleucine to 100 ug/ml. Alternatively, starvation was imposed by the addition of serine hydroxamate at 100 ug/ml (Laffler and Gallant, 1974).

At timed intervals following the onset of starvation, aliquots of culture were removed into an equal volume of 10% trichloroacetic acid. The precipitate, separated by filtration on GF/A filters, was washed with 3 x 3 mls of 5% TCA at 0°. After drying, the filter was counted in toulene Omnifluor (New England Nuclear) mixture.

Downshift experiments

Carbon source depletion was imposed on cultures growing in Tris-minimal (0.1% glucose) by the addition of the non-metabolizable glucose analog α-methylglucoside (αMG) to a final concentration of 2.0%. The analog is a competitive inhibitor of glucose uptake in *E. coli* (Kessler and Rickenberg, 1963), but does not appear to have any other deleterious

effects on the cell. This is inferred from the observation that cells growing in the presence of other carbon sources (*e.g.*, glycerol, fructose) are not affected by the analog (Hansen *et al.*, 1975). The downshift condition is reversed by the addition of glucose to a final concentration of 2.0%.

Thin layer chromatography

Thin layer chromatography was performed according to the procedures described by Cashel *et al.* (1969). Poly(ethylene)imine (PEI) cellulose thin layer plates (Brinkman) were prepared for use by soaking in 50% methanol containing 0.05% Triton-X for 30 min, and then drying at room temperature. Aliquots (10-30 ul) of the samples were applied without intermediate drying along the origin 2.5 cm from the lower edge of the chromatogram. After drying at room temperature, the chromatograms were generally desalted by soaking in methanol for 15 min, then redried at room temperature. The chromatograms were developed without preequilibration by ascending development in a closed chamber.

The identification of radioactive materials was confirmed by their comigration with authentic standards. The ^{32}P labeled materials were visualized by exposing an X-ray film (Kodak BB-1) to the dried chromatograms for an appropriate period (about 12 hrs) for 5000 CPM), and developing the exposed film. The radioactive areas of the chromatogram were quantitated by cutting out the appropriate area, and counting it in toluene scintillation fluid.

Estimation of nucleotide pools

Intracellular nucleotide pools were determined by separating radioactively labeled compounds on PEI thin layers, and counting the cutouts.

Exponential cultures (A_{450}≃0.2) were grown in Tris minimal medium at 32°. The cultures were supplemented with $^{32}PO_4$ (250 uCi/umole), and grown for at least one doubling in order to ensure equilibration of the phosphate pools. Extracts were prepared by adding formic acid to a final concentration of 1.0 M. After 30 min at 0°, the extracts were cleared by centrifugation, and 10-30 ul aliquots of the supernatant were chromatographed on PEI cellulose by ascending development to 17 cm above the origin. The nucleotides ATP, GTP, ppGpp, and pppGpp are well resolved by one dimensional development in 1.5 M KH_2PO_4 (pH 3.4) (Figure 2). Absolute incorporation of ^{32}P was calculated from counts obtained with 2 ul aliquots of the cultures which were spotted directly on PEI cellulose cutouts, dried, and counted. The pool size is expressed as pmoles of nucleotide per optical density unit of cells at 450 nm (*i.e.*, pmole/A_{450}). One A_{450} unit corresponds to approximately 4×10^8 cells/ml (Fiil *et al.*, 1972).

For mass screening experiments, 75 ul cultures were grown in 12 x 75 mm culture tubes, starved by the addition of 5 ul of an amino acid analog, and extracted by the addition of 20 ul of 5.0 M formic acid.

Buffers

Buffer-A: contained 50 mM Tris-OAc (pH 8.0), 15 mM $Mg(OAc)_2$, 60 mM KOAc, 27 mM NH_4OAc, 1 mM dithiothreitol (DTT) and 0.2 mM Na_2EDTA (Cashel, 1974).

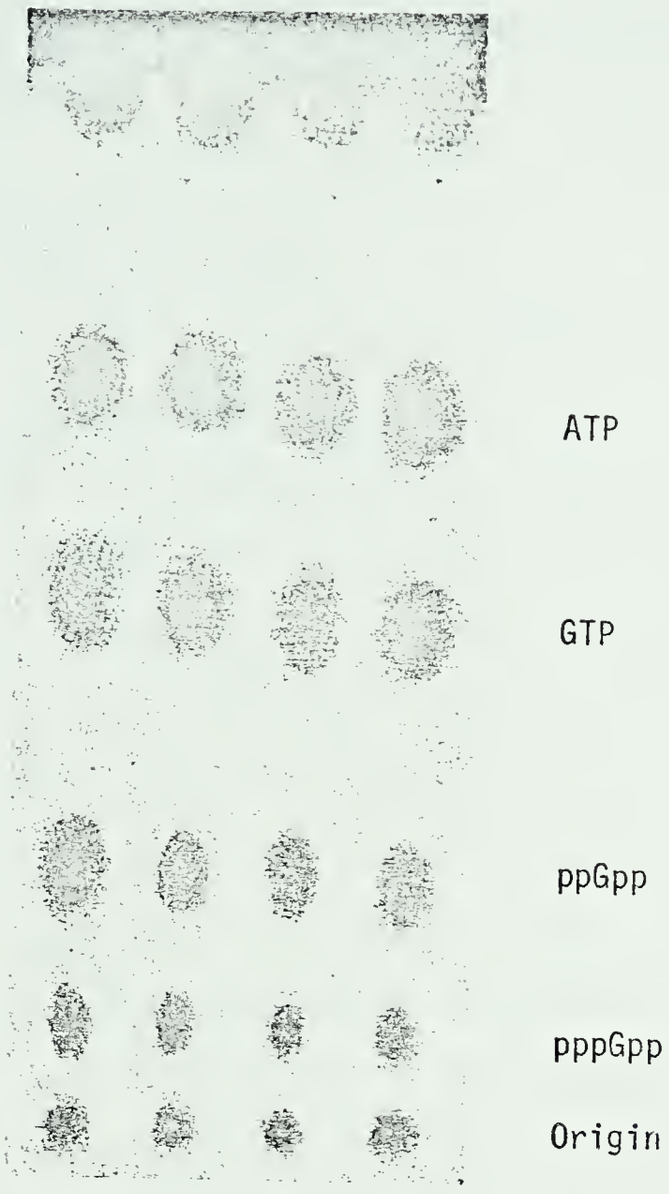

Figure 2 - Typical separation of various nucleotides on PEI cellulose. Formic acid extracts of $^{32}PO_4$-labelled cultures were applied to a PEI cellulose thin layer plate. The chromatogram was developed with 1.5 M KH_2PO_4, dried, and autoradiographed.

Buffer-B: contained 50 mM Tris-OAc (pH 7.4), 10 mM $Mg(OAc)_2$, 1 mM DTT and 0.2 mM Na_2EDTA.

Preparation of ^{32}P-labeled nucleotides

Uniformly labeled [^{32}P]GTP was prepared from heavily labeled cultures according to the procedures described by Cashel and Kalbacher (1970). Small cultures (1 or 2 ml) were grown in Tris minimal medium containing $^{32}PO_4$ (500 uCi/umole) for one doubling prior to extraction with 0.2 volumes of 5.0 M formic acid. The acid extracts were applied to PEI cellulose TLC plates (1 ml of extract per 20 cm of origin), developed with 1.5 M KH_2PO_4, dried, and eluted with 3 ml of 4.0 M LiCl after autoradiographic localization. The eluate was neutralized with NH_4OH, and the precipitate was collected by centrifugation. After two washes with ethanol, the precipitate was resuspended in a minimal volume of 1.0 M formic acid, and adsorbed to 20 mg of acid washed Norit-A. The charcoal was washed with 50 ml of H_2O to remove Pi, and the GTP was then eluted with 15 ml of 2.8% NH_4OH. The eluate was flash-evaporated at 50°, resuspended in a small volume of distilled water, and stored frozen at -40°. The uniformity of labeling was determined by hydrolysis with repurified phosphodiesterase-1, and chromatography of the reaction products.

Adenosine 5'-[β-^{32}P] triphosphate was prepared according to the enzymatic procedure described by Leung and Yamazaki (1977). The method exploits a coupled reaction in which, in the first step, polynucleotide phosphorylase catalyzes the formation of [β-^{32}P]ADP from polyadenylate and $^{32}PO_4$ (carrier free). In the second step,

pyruvate kinase phosphorylates ADP to ATP at the expense of phosphoenolpyruvate. The ATP is subsequently separated from the other components of the reaction by chromatography on PEI cellulose. The method routinely permits recovery of 60% of the input radioactivity as specifically labeled ATP of high specific activity.

Preparation of ribosomes and enzyme extracts

The procedure described below is generally similar to the method described by Nirenberg and Matthaei (1961) for the preparation of the components of a cell-free translation system.

Cultures were grown to mid log phase in Tris medium supplemented with 0.4% glucose and 0.8% nutrient broth. The cells were harvested by centrifugation, washed with 100 mM Tris-HCl (pH 7.5), and frozen at -40° until use. Extracts were prepared by grinding the cell pellets with alumina in buffer-A. DNAase (Worthington) was added to 4 ug/ml, and the extract was cleared twice at 30,000 x *g* for 30 min. The upper 80% of the supernatant was recovered by aspiration; this constituted the S-30 extract. These S-30 extracts were subsequently centrifuged in a Beckman Ti-50 rotor for 3 hr at 100,000 x *g* (39,000 rpm). The upper 80% of the supernatant was retained; this constituted the essentially ribosome-free S-100.

Low salt washed ribosomes were prepared by rinsing the 100,000 x *g* pellet with buffer-A, gently resuspending the pellet in the same buffer, and then centrifuging as before. This procedure was repeated four times in order to remove GTP*ase* and pppGpp*ase* activities from the ribosomes. The final preparation was resuspended in buffer-A at a concentration of 250 A_{260} units/ml, and stored at 4° until use. The

yield was generally about 700 A_{260} units per litre of culture.

In vitro synthesis of pppGpp and ppGpp

The ribosome dependent *in vitro* reaction, first described by Haseltine *et al.* (1972) and subsequently modified by Cashel (1974), was employed for both analytical and preparative scale synthesis of pppGpp.

For relatively large scale preparations, the reaction contained low salt washed ribosomes in buffer-A at 120 A_{260} units/ml, 2 mM GTP, 4 mM ATP, 3 mM PEP, and pyruvate kinase (36 units/ml, ammonium sulfate free) in a volume of 30 ml. After 8-10 hr at 23°, the ribosomes were precipitated from the reaction mixture by the addition of 1/40 volume of 90% formic acid, and removed by centrifugation (15,000 x *g* for 10 min at 4°). The supernatant was made 1.0 M in LiCl by the addition of the solid salt, then the nucleotides were precipitated by the addition of 5 volumes of ethanol at -20°. The precipitate was recovered by centrifugation (15,000 x *g* for 10 min), washed once with ethanol, then dried under an air stream. After drying, the precipitate was resuspended in the minimal volume of 0.5 M sodium formate (pH 3.4), made 50 mM in Tris-Cl, and adjusted to pH 7.4 with NH_4OH. The solution was applied to a 1.2 x 40 cm DEAE Sephadex-A25 column, previously equilibrated with a solution of 50 mM Tris-Cl + 0.15 M LiCl, and eluted with a 400 ml linear gradient of LiCl (0.15-0.5 M) in the same buffer with a flow rate of 20 ml/hr at 23°. The absorbance at 254 nm was monitored with an LKB recorder. A typical absorbance profile is

illustrated in Figure 3. Fractions containing pure pppGpp (as identified by thin layer chromatography), were pooled, flash-evaporated at 50° to a thick syrup, and precipitated with 5 volumes of ethanol (-20°). The precipitate was collected by centrifugation, washed twice with ethanol and air dried. The nucleotide was stored in buffer-A at -40°. The amount of the nucleotide was determined by assuming the same molar extinction coefficient as ppGpp (12,700 in 0.1 N HCl at 257 nm; Cashel and Kalbacher, 1970). The purity was assessed as greater than 90% by measuring the peak areas of ultraviolet absorption following rechromatography on DEAE Sephadex (ppGpp was the only significant contaminant).

The preparation of 3'-[α-^{32}P]pppGpp (Sy and Lipmann, 1973) was accomplished by combining the completed reaction mixture of Leung and Yamazaki (1977) with the components of the (p)ppGpp biosynthetic reaction. This approach resulted in the recovery of approximately 30% of the $^{32}PO_4$ as (p)ppGpp.

Fractionation and assay of pppGppase and GTPase activities

Twenty-five milligrams of S-100 protein in buffer-A was applied to a 1.2 x 33 cm DEAE Sephadex column (in acetate form), previously equilibrated with 0.1 M KOAc in buffer-B. The column was eluted with a 400 ml linear gradient (0.1-0.5 M) of KOAc in buffer-B at 10 ml/hr at 4°. The eluate was collected as 90 fractions, which were then dialyzed against several hundred volumes of buffer-A in order to equalize the salt concentration.

The ribosome-independent nuclease activity of each fraction was assayed in a 30 ul reaction containing 0.4 umole of ^{32}P-labeled nucleo-

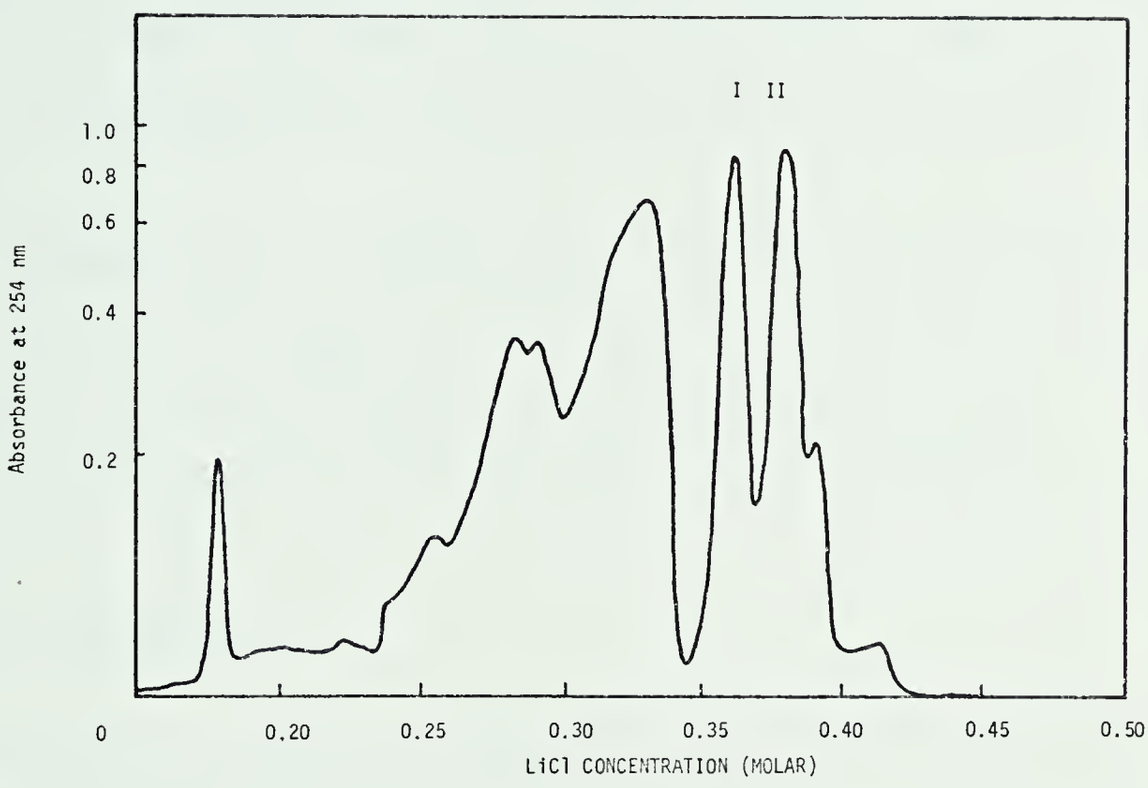

Figure 3 - Typical elution profile of ppGpp and pppGpp from a DEAE Sephadex column.
The general procedure is described in the text. Peaks I and II were identified as ppGpp and pppGpp, respectively, by thin layer chromatography.

tide (in 10 ul of buffer-A), and 20 ul of extract. Following a period of incubation at 32°, the reaction was terminated with 5 ul of 5 M formic acid. Aliquots (20 ul) of the reaction mixtures were applied to PEI cellulose, and the chromatograms were developed in 1.5 M KH_2PO_4. The nucleotides were visualized under short wavelength ultraviolet light, and the spots were cut out and counted. Under these conditions, there is no significant spontaneous degradation of GTP, ppGpp, or pppGpp for at least 80 min. Crude extracts were assayed in a similar manner.

Ribosome-dependent nuclease activity was measured in the same way, except that 2.5 A_{260} units of low salt washed ribosomes were included in the reaction mixture. The ribosome precipitate was removed by centrifugation before the sample was chromatographed.

Protein concentration

Protein concentration was determined with the Folin-Ciocalteau reagent using bovine serum albumin as the standard.

RESULTS

Isolation of mutants which accumulate elevated levels of pppGpp

The objective was to isolate mutants defective in the hydrolysis of pppGpp. Since there were several conflicting hypotheses concerning the effects of such a mutation, the screening procedure was necessarily simple and direct.

From a nitrosoguanidine mutagenized culture of the strain CS251 (F⁻ *lacZ metG hisC thyA strA xyl mtl argH rif*^{r} *glpK nalA*), 500 randomly selected colonies were transferred to master plates, and screened for the levels of the MS-nucleotides (ppGpp and pppGpp) under conditions of amino acid starvation. Cultures growing at 32° in $^{32}PO_4$-supplemented Tris minimal were shifted to 42° for 15 min, subjected to valine-induced isoleucine starvation for 6 min, and extracted with formate. The intracellular concentration of the MS-nucleotides was measured by PEI cellulose chromatography of the acid extracts, as described in *Materials and Methods*. The temperature shift was included in the protocol to permit the recovery of temperature sensitive mutations.

Of the 409 strains examined in this manner, 3 strains (designated MS50, MS217 and MS240) were found to have abnormally high levels of pppGpp, but approximately normal levels of ppGpp. The working hypothesis, formulated at this point, was that these mutants were accumulating increased amounts of pppGpp because of a defect in the hydrolysis of the nucleotide. The mutants were, therefore, tentatively designated as *gpp* mutants (*g*uanosine *p*enta*p*hosphatase).

The *gpp* phenotype, under conditions of amino acid starvation, is

illustrated for a pair of otherwise isogenic strains in Figures 4 and 5. It can be seen that the level of pppGpp is significantly higher in the mutant than in the wild type strain, whereas the level of ppGpp is similar in both. Although the response is illustrated here for only two amino acids, it has also been observed during methionine and tryptophan starvation. It, therefore, appears to be a general response to starvation for any amino acid.

Following the relaxation of starvation conditions, the level of pppGpp declines very rapidly to the basal level in both the mutant and the wild type (Figure 4). This response appears to be contradictory to the hypothesis that the increased accumulation of pppGpp is due to a defect in the rate of turnover. It must be noted however, that the levels of the MS-nucleotides observed during starvation reflect an equilibrium between net synthesis and decay. Lund and Kjeldgaard (1972) have calculated that amino acid starved cells, in the steady state, form and turnover 10^6 molecules of ppGpp/min/cell. Thus, a relatively slight change in the rate of pppGpp hydrolysis might account for the increased level of accumulation, and yet be undetectable by measuring the rate of decay following the release of starvation conditions. It should also be noted that, in the *gpp* mutant, pppGpp accumulates and decays more rapidly than ppGpp. This effect is particularly pronounced in the case of the serine hydroxamate induced serine starvation (Figure 5), where the onset of starvation appears to be less rapid than the valine induced isoleucine starvation (Figure 4). A similar, but less pronounced, response in the wild type strain has been observed by Kari *et al.* (1977), and interpreted as evidence that pppGpp is a precursor of ppGpp.

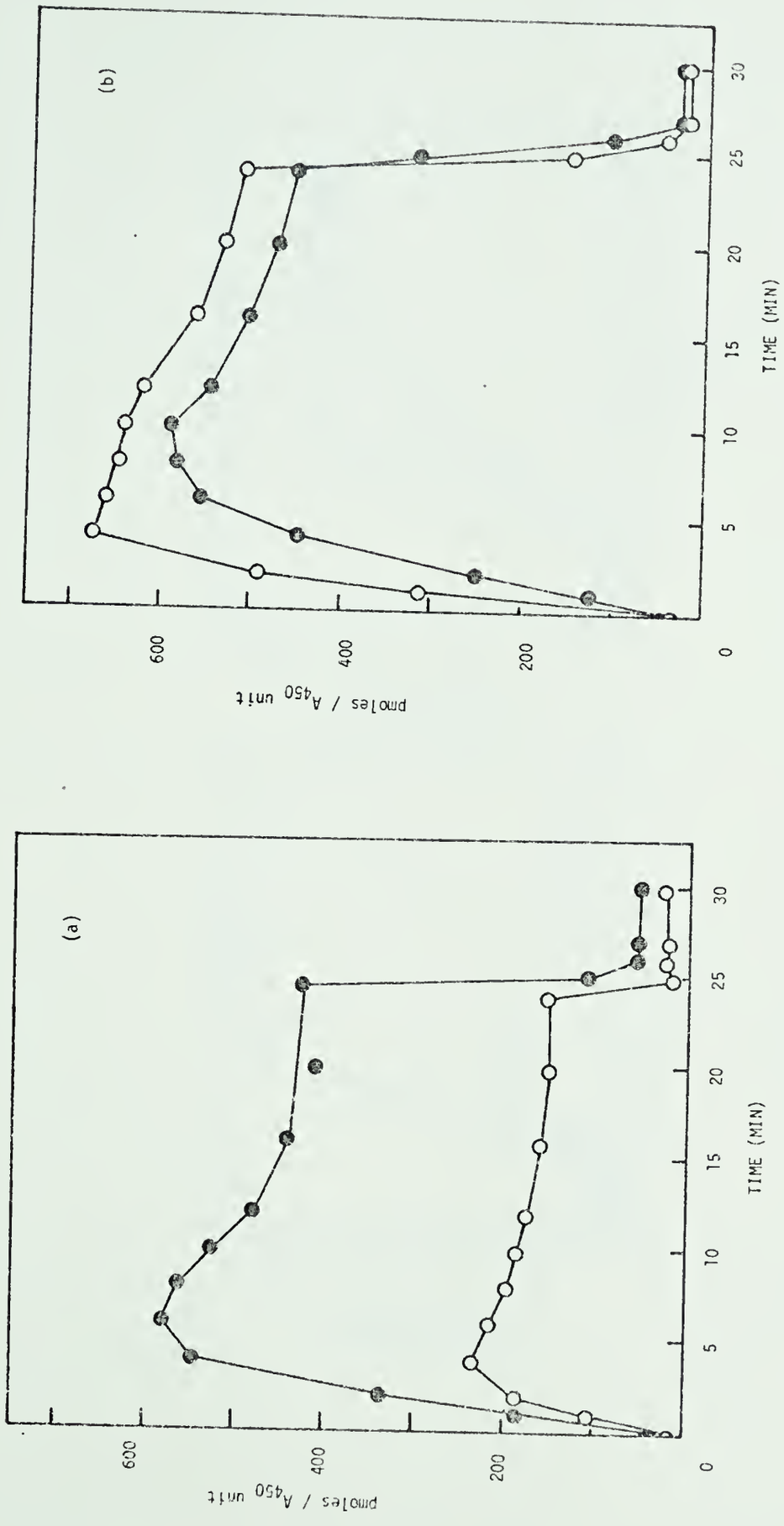

Figure 4 - Valine-induced accumulation of MS-nucleotides in the wild type and a *gpp* mutant.
Cells grown in Tris minimal at 32° were subjected to valine-induced isoleucine starvation. At the indicated intervals, samples of the culture were assayed for the levels of ppGpp and pppGpp. At 24 min, starvation was alleviated by the addition of L-isoleucine to a final concentration of 100 ug/ml. (a) CS417 (*gpp*$^+$ *relA*$^+$ *spoT*$^+$), (b) CS416 (*gpp*$^-$ *relA*$^+$ *spoT*$^+$). Symbols: (•) ppGpp, (o) pppGpp.

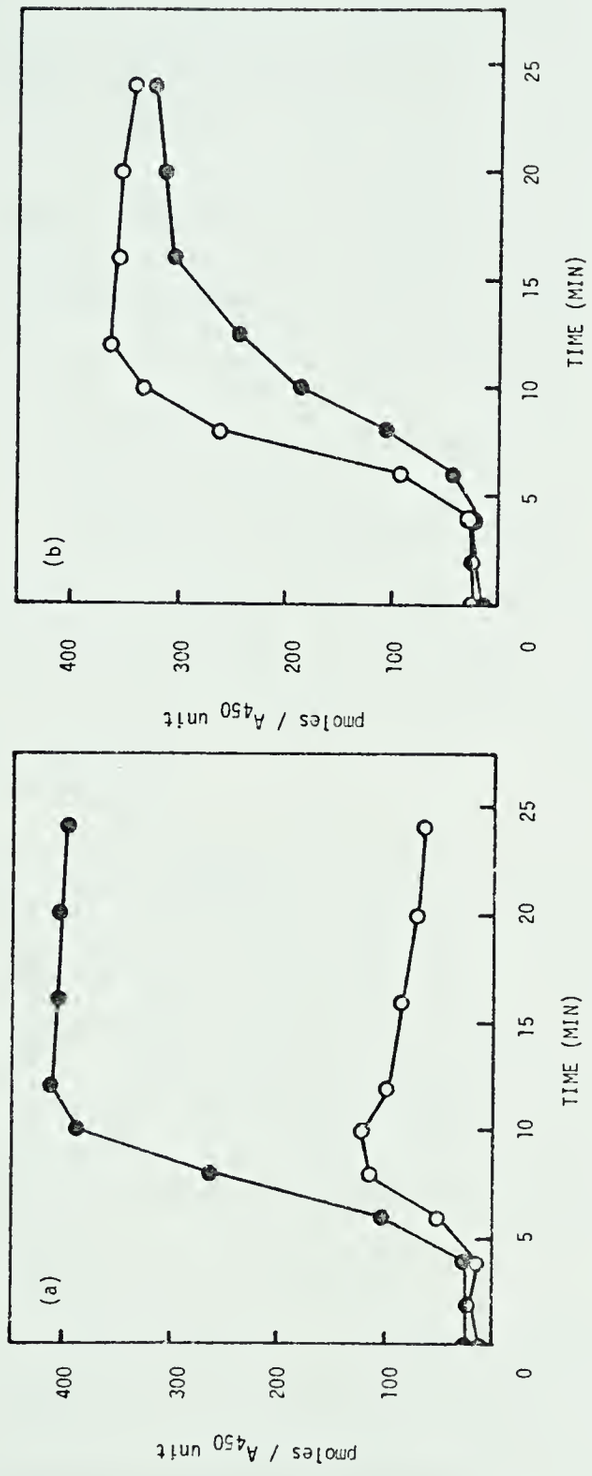

Figure 5 - Serine hydroxamate induced accumulation of MS-nucleotides in the wild type and a *gpp* mutant.
The conditions for the assay are described in Figure 4 except that serine hydroxamate was used to effect serine starvation.
(a) CS417 (*gpp*$^+$ *relA*$^+$ *spoT*$^+$), (b) CS416 (*gpp*$^-$ *relA*$^+$ *spoT*$^+$). Symbols: (●) ppGpp, (○) pppGpp.

The isoleucine starvation induced changes in the ATP and GTP pools are illustrated in Figure 6. The rate and extent of the decline of the GTP pool in the *gpp* mutant appears to be greater than in the wild type strain. The decline in the GTP pool following amino acid starvation has been attributed to inhibition of IMP dehydrogenase by ppGpp (Gallant *et al.*, 1971). Thus, the increased (p)ppGpp pool in the *gpp* mutant might bring about a proportionately greater degree of inhibition of IMP dehydrogenase, with the result that the rate of GTP synthesis is lower in the mutant during amino acid starvation. Alternatively, the *gpp* mutants may place a greater demand upon the GTP pool as a precursor of (p)ppGpp. The rapid increase in the GTP pool following the release of starvation has been attributed to recycling of the guanosine 5'-polyphosphate moiety of (p)ppGpp (Kari *et al.*, 1977)

Mapping the gpp mutation

The mutant strain MS240 (F^- *gpp-1 lacZ metG hisC thyA strA xyl mtl argH* rif^r *glpK nalA*), which exhibited the most extreme phenotype of the three original *gpp* mutants, was chosen for mapping studies. Since no other phenotype could be ascribed to the *gpp* mutation, recombinants were scored by measuring the accumulation of the MS-nucleotides following isoleucine starvation.

A series of short duration conjugal crosses, involving several different Hfr strains, was undertaken in order to test for linkage between the *gpp* mutation and the nutritional markers in MS240. Out of 14 $argH^+$ str^r recombinants obtained from a 30 min mating between Hfr R1 (*metB relA*) and MS240, 2 recombinants had MS-nucleotide levels characteristic of the wild type. Also, out of 30 xyl^+ str^r recombinants recovered from a 30 min mating between Hfr KL209 (*mal supE*) and MS240, 20

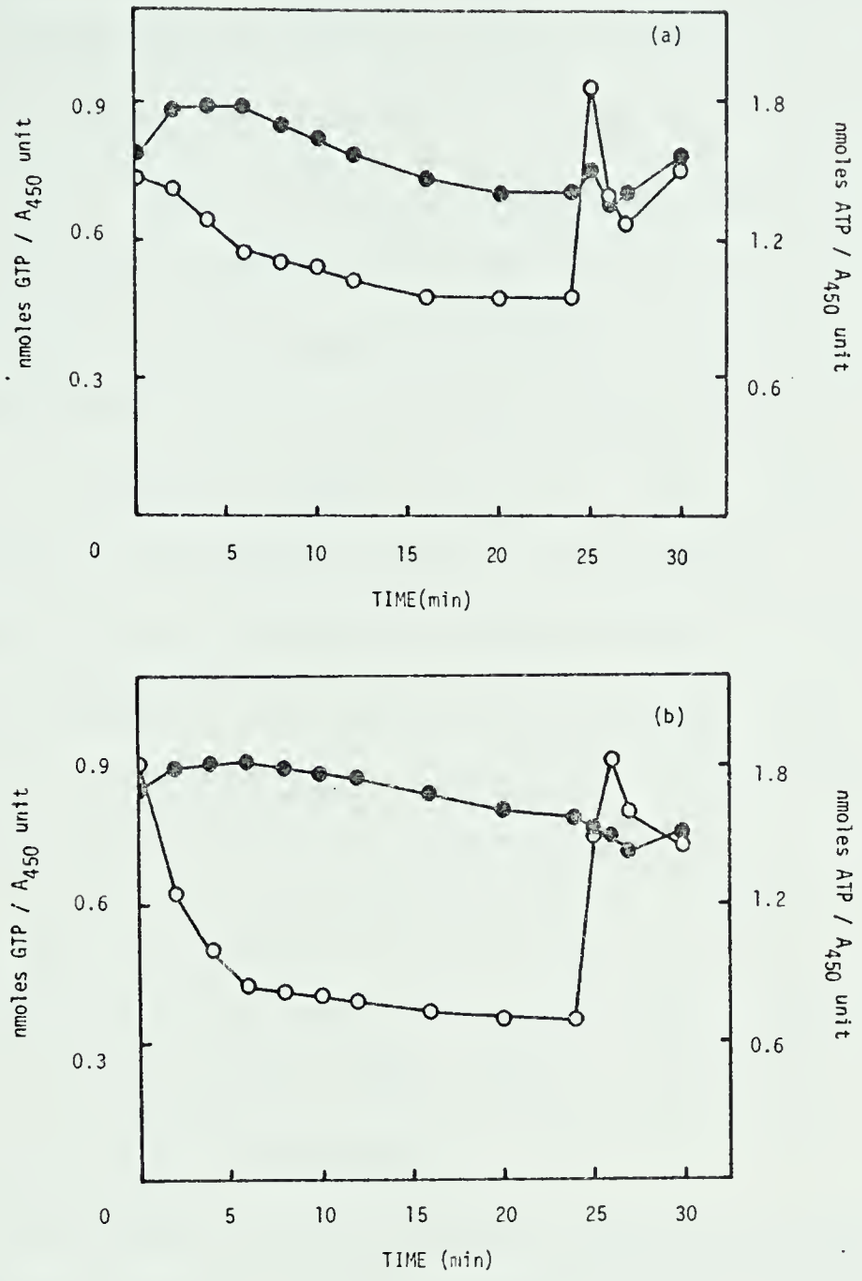

Figure 6 - Response of ATP and GTP pools to amino acid starvation of a wild type strain and a *gpp* mutant. The conditions of the assay are as described in the legend to Figure 4. (a) CS417 (*gpp*$^+$ *relA*$^+$ *spoT*$^+$), (b) CS416 (*gpp*$^-$ *relA*$^+$ *spoT*$^+$). Symbols: (•) ATP, (o) GTP.

were wild type for the *gpp* locus. These results suggested a location for the *gpp* mutation in the relatively short *ilv-malB* region of the chromosome, proximal to the points of origin of the two Hfr strains.

Since the point of origin of Hfr Ra-2 (*mal sfa*) bisects this region of the map, a short (30 min) mating was performed between Ra-2 and MS240. All of the 32 argH$^+$ str^r recombinants obtained from this cross were of the mutant phenotype. This negative result suggested a location in the *ilv-rha* region, distal to the point of origin of Ra-2 and proximal to the point of origin of Hfr Rl. Since a number of auxotrophic markers are available for this three minute interval, a transductional cross was performed. A P1*vir* lysate of MS240 was used to transduce KL266 (F$^-$ *leu ara proC lacZ hisF cysC thyA str spc xyl mtl metE thi*). Of the 15 metE$^+$ transductants recovered from this cross, 3 had MS-nucleotide levels characteristic of the *gpp* mutants. Linkage to *ilvD* was also demonstrated by a cross in which the strain CS401 (F$^-$ *ilvD glpK metB argH malA mtl thi strA*) was transduced with a P1*vir* lysate of the *gpp* mutant MS240. Of the 32 ilvD$^+$ transductants recovered from this cross, 20 were gpp$^-$.

In order to position the *gpp* locus with respect to adjacent genes, several three-point transductions were performed. The *gpp-1* mutation from MS240 was first transduced into the strain CS403 (F$^-$ *gpp-1 glpK metB argH malA mtl thi strA*), by selecting an ilvD$^+$ transductant of CS401. This was accomplished in order to eliminate other closely linked mutations which generally result from nitrosoguanidine mutagenesis (Guerola *et al.*, 1971). A P1 lysate of CS403 was subsequently used to transduce CS410 (F$^-$ *hisF cysC thyA str spc xyl ilvD metE thi*) to metE$^+$, with *ilv* and *gpp* as unselected markers. The results

of this cross, presented in Table 2, indicate that the *gpp* locus is within the *ilvD-metE* interval. This is inferred from the observation that all those recombinants which were ilvD$^+$ were also gpp$^-$, but that not all of the gpp$^-$ recombinants were ilvD$^+$. Similarly, the position of the *gpp* locus relative to *cya* and *ilv* was determined by a second three-point cross. In this instance, a P1 lysate of the strain CS414 (F$^-$ *hisF cysC thyA str spc xyl* Δ*cya metE thi*) was used to transduce CS415 (F$^-$ *hisF cysC thyA str spc xyl ilvD gpp thi*) to ilv$^+$, and the *gpp* and *cya* phenotypes were scored. The results of this cross, presented in Table 3, indicate the gene order *ilvD gpp cya*. This sequence is inferred from the observation that, all those recombinants which were cya$^-$ were also gpp$^+$, whereas many of the gpp$^+$ recombinants remained cya$^+$. The results of these mapping experiments are summarized in Figure 7.

The gpp mutation is recessive

The recessive nature of the *gpp* mutation was demonstrated by introducing an F' episome, carrying a wild type allele of the *gpp* gene, into a *recA* derivative of a *gpp* mutant. The F' strain AB1206 (F14 *ilv-argH/ Δ(ilv-argH) pro lac supE gal his strA thi*) was crossed with the *gpp* mutant CS409 (F$^-$ *recA strA spc km^r xyl gpp metE thi*). Ten met$^+$ Km$^+$ (kanamycin resistant) merodiploids were screened for the *gpp* phenotype, and found to be wild type. The presence of the F14 episome in these putative merodiploids was confirmed by their sensitivity to the male-specific phage R17.

Table 2

Mapping the *ilvD*, *gpp*, and *metE* genes by a three-point transductional cross.

Selected marker	Recombinant markers	Number of recombinants
$metE^+$	ilv^+ gpp^-	29
$metE^+$	ilv^- gpp^-	5
$metE^+$	ilv^+ gpp^+	0
$metE^+$	ilv^- gpp^+	71

A P1*vir* lysate, prepared on a *gpp* donor strain CS403 (F^- *gpp glpK metB argH malA mtl thi strA*), was used to transduce an *ilvD metE* recipient CS410 (F^- *hisF cysC thyA strA spc xyl ilvD metE thi*) to $metE^+$. The allelic state of the *gpp* gene in the recombinants was determined in liquid cultures by measuring the intracellular levels of the MS-nucleotides following methionine starvation.

Table 3

Ordering of the *ilvD*, *gpp*, and *cya* genes by a three-point transductional cross.

Selected marker	Recombinant markers	Number of recombinants
ilv^+	gpp^- cya^+	17
ilv^+	gpp^+ cya^+	58
ilv^+	gpp^+ cya^-	37
ilv^+	gpp^- cya^-	0

A P1*vir* lysate prepared on a *cya* donor strain CS414 (F^- *hisF cysC thyA str spc xyl Δcya metE thi*) was used to transduce an *ilvD gpp* recipient CS415 (F^- *hisF cysC thyA str spc xyl ilvD gpp thi*) to ilv^+. Transductants were scored for the *cya* phenotype on MacConkey agar containing lactose and maltose. The *gpp* phenotype was scored in liquid cultures, by determining the intracellular levels of the MS-nucleotides following isoleucine starvation.

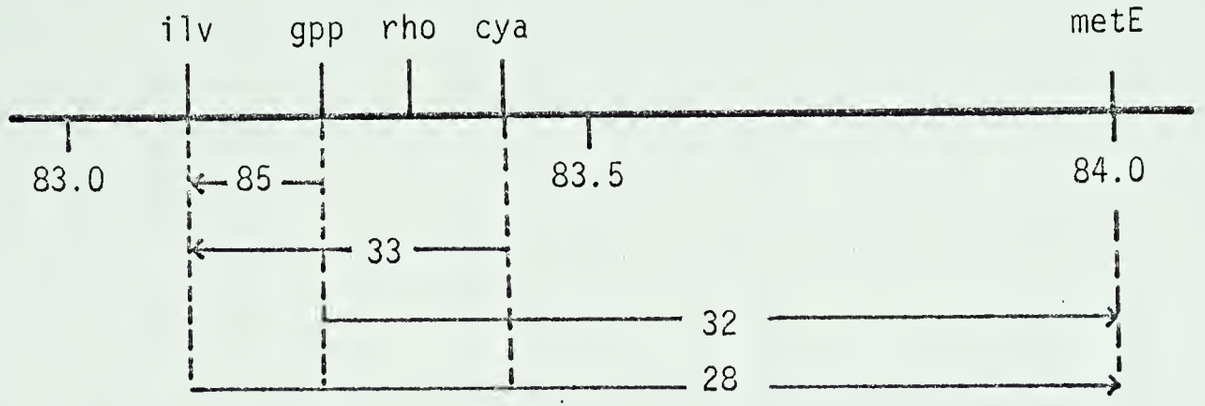

Figure 7 - Linkage map of the *ilv-metE* region of the *E. coli* chromosome showing the relative positions and cotransduction frequencies of various genes. The positions of all loci except *gpp* are as reported by Bachmann *et al.* (1976). The numbers directly below the map denote the position (in min.) of the segment on the *E. coli* chromosome. The numbered arrows below the map indicate the frequency of cotransduction (expressed as percentages), and the arrowheads point to the selected marker.

Isolation of gpp mutants by localized mutagenesis

As previously noted, the three original *gpp* mutants show a rapid rate of pppGpp decay following the release of amino acid starvation. It was, therefore, of interest to determine whether *gpp* mutants of more extreme phenotype could be isolated. For this reason, a second attempt at mutant isolation was undertaken. In order to increase the recovery of *gpp* mutants, a technique of localized mutagenesis (Hong and Ames, 1971) was employed. This method takes advantage of the observation that nitrosoguandine induces closely linked multiple mutations (Guerola *et al.*, 1971). Thus, by selecting for revertants of an auxotrophic mutation, one can selectively mutagenize the area of the chromosome adjacent to the site of the reversion.

From 144 nitrosoguanidine induced ilv^+ revertants of the strain CS401 (F^- *ilvD glpK metB argH malA mtl strA* sup_{am} *thi*) 6 mutants with elevated levels of pppGpp were isolated by the same screening procedure used for the isolation of the original *gpp* mutants. These mutants, designated MS551, 552, 556, 573, 631, and 641, exhibited phenotypes which were generally indistinguishable from the original three mutants. These 6 mutants, like the original 3 mutants, were all found to carry mutations which were cotransducible with *metE* and which appeared to be responsible for the high level of pppGpp accumulation during amino acid starvation. This was demonstrated by transducing KL266 (F^- *leu ara proC lacZ hisF cysC thyA strA spc xyl mtl metE thi*) to $metE^+$ with P1 lysates of the mutants and recovering, in each case, recombinants which exhibited the *gpp* phenotype. It is, therefore, tentatively concluded that all nine mutations are allelic within the *gpp* locus, or mutations in closely linked genes of similar function.

This experiment also resulted in a series of strains which are isogenic except for a small region surrounding the *gpp* locus. These strains are designated in Table 1 as CS425-432. The *gpp* phenotype for several of these strains and an appropriate wild type control, is illustrated in Figure 8. It can be seen from these results that the four *gpp* mutations produce a very similar phenotype. The slight variation in the maximal level of ppGpp accumulation among the various strains is probably due to some variation in the experimental conditions, and is not considered significant in this instance. It should also be noted from these results that the *gpp* phenotype is relatively insensitive to differences in the genetic background of the strains (*i.e.*, the *gpp-1* allele is present in strains CS416 (Figure 8b) and CS403 (Figure 8c)).

The isolation of the additional *gpp* mutants was not informative. The unfortunate choice of CS401 (which carries an amber suppressor) as a parental strain, precluded the isolation of *amber* mutants. Also, none of the mutants had a temperature dependent phenotype.

In vitro synthesis of MS-nucleotides

Two simple, alternate hypotheses could account for the increased level of pppGpp accumulation in the *gpp* mutants. Either there is an increased rate of synthesis, or a decreased rate of degradation. The *in vitro* ribosome idling reaction, described by Haseltine *et al.* (1972), presented an attractive means of testing the first hypothesis.

Low salt washed ribosomes were prepared from the strains CS416

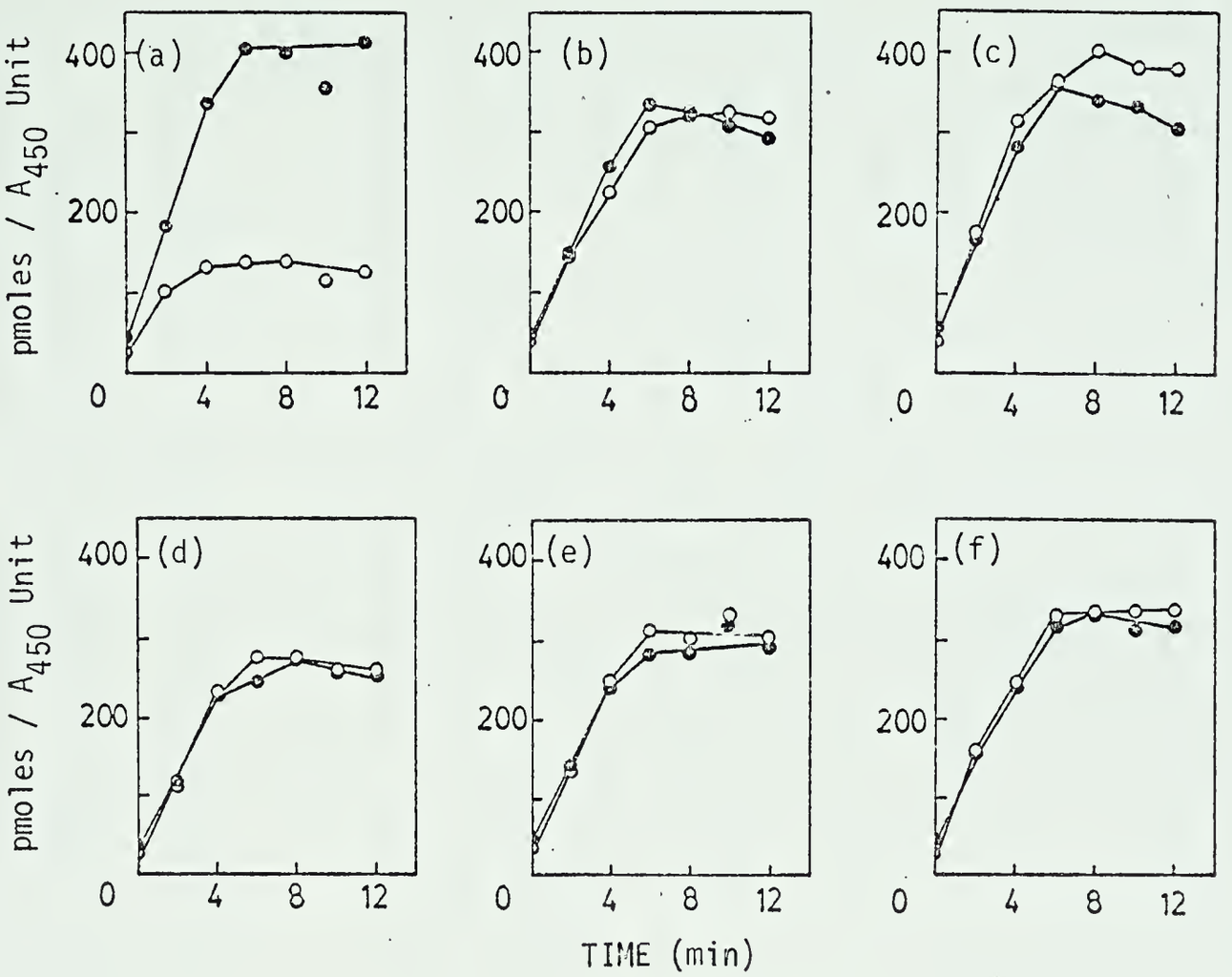

Figure 8 - Nucleotide accumulation in various *gpp* mutants following amino acid starvation. Exponential cultures, growing in Tris minimal at 32°, were subjected to valine-induced isoleucine starvation at time 0. At the indicated intervals, samples were removed for nucleotide pool measurements, as described in *Materials and Methods*. (a) KL266 (gpp^+), (b) CS416 (*gpp-1*), (c) CS403 (*gpp-1*), (d) CS425 (*gpp-2*), (e) CS426 (*gpp-3*), and (f) CS427 (*gpp-4*). Symbols: (●) ppGpp, (o) pppGpp.

(*gpp-1*...) and CS417 (*gpp*$^{+}$...), which were derived by transduction from a common *relA*$^{+}$ parent strain (Table 1). The ribosomes were washed three times, rather than five, in order to allow expression of any difference between the strains with respect to a ribosome-associated pppGpp*ase* activity. The MS-nucleotide synthetic ability of the ribosomes was assessed by incubating the ribosomes with ATP and GTP, and then separating the products of the reaction by PEI cellulose chromatography. The results of this experiment, presented in Figure 9, indicate that the ribosomes of the two strains are indistinguishable with respect to MS-nucleotide synthetic ability. It was, therefore, tentatively concluded that the *gpp* mutation does not directly affect the rate of synthesis of pppGpp. A major caveat to this conclusion is that the *in vitro* conditions may not correspond to the *in vivo* conditions in some important way. Such an explanation has been invoked, for example, to explain the observation that *spoT* mutants, which do not synthesize significant levels of pppGpp *in vivo*, synthesize normal levels of this nucleotide *in vitro* (De Boer *et al.*, 1977).

In vitro evidence that gpp mutants are defective in a pppGppase activity

It has previously been reported that pppGpp is unstable in crude extracts which are capable of supporting translation (Yang *et al.*, 1974). It has also been noted that, in the presence of ribosomes, the elongation factors G (EF-G) and T (EF-T) are capable of converting pppGpp to ppGpp (Hamel and Cashel, 1973; Cochran and Byrne, 1974). It was, therefore, reasonable to examine the effects of the *gpp* mutations on the *in vitro* degradation of pppGpp.

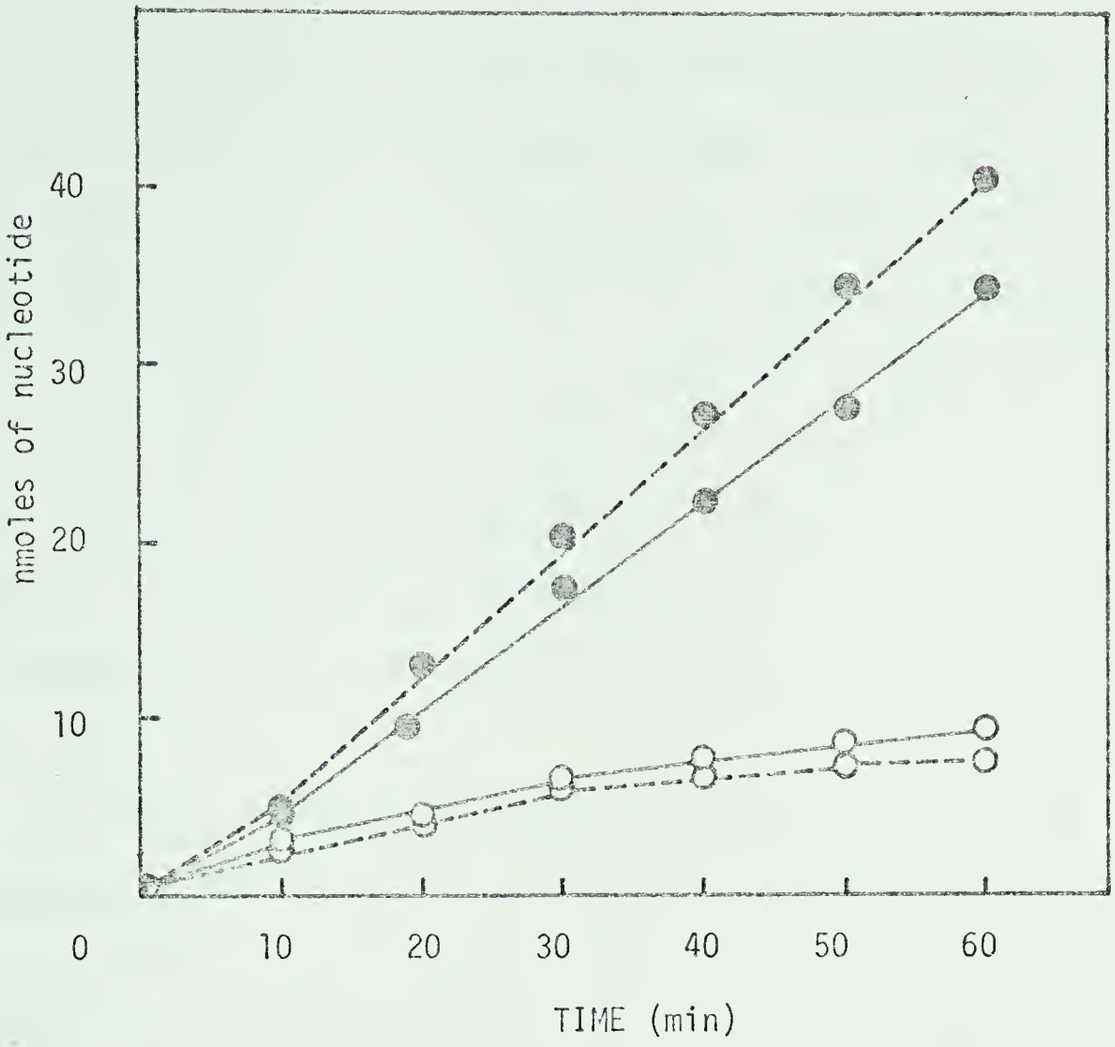

Figure 9 - *In vitro* synthesis of ppGpp and pppGpp by ribosomes from the wild type and a *gpp* mutant. The products of the reaction catalyzed by ribosomes from the wild type strain CS417, are indicated by dashed lines (---). The products of the reaction catalyzed by ribosomes from the *gpp-1* mutant strain CS416, are indicated by the solid lines (——). The open (o) and closed (●) symbols represent pppGpp and ppGpp respectively. The 250 ul reaction contained 24 A_{260} units of low salt washed ribosomes, 0.125 umoles GTP, and 0.375 umoles 5'-[β-^{32}P]ATP (58.8 uCi/umole). The final concentration of buffer and salts was the same as in buffer-A (*Materials and Methods*). The reaction was incubated at 32°. At the indicated intervals, 30 ul samples were withdrawn into 15 ul of 2 M formate, and chromatographed on PEI cellulose thin layer plates.

Crude S-30 extracts, potentially capable of supporting *in vitro* translation, were prepared from the wild type strain CS251 and the *gpp-1* mutant MS240. These extracts were assayed for pppGpp*ase* activity by incubating an aliquot of the extract with [^{32}P]pppGpp and chromatographing the reaction products on PEI cellulose. From the results of this assay, presented in Figure 10, it can be seen that the *gpp-1* mutant has a substantially lower level of pppGpp*ase* activity than the wild type. As previously noted, ppGpp appears to be the major product of the reaction, and is relatively stable under these conditions (Yang *et al.*, 1974).

In order to distinguish between ribosome-dependent versus ribosome-independent pppGpp*ase* activity, ribosome-free S-100 extracts of a wild type strain, a *spoT* mutant, and three independent *gpp* mutants were prepared. The S-100 extracts were then assayed for pppGpp*ase* activity as before. The results of this experiment, presented in Figure 11, clearly show that the *gpp* mutants are completely deficient in a ribosome-independent pppGpp*ase* activity.

The foregoing conclusion is substantiated by a series of experiments in which the various pppGpp*ase* activities of the wild type and the *gpp-1* mutant were fractionated on a DEAE Sephadex column. S-100 extracts of the *gpp-1* mutant CS416 and the wild type strain CS417 were prepared and chromatographed under identical conditions as described in *Materials and Methods*. The fractions were then assayed for pppGpp-*ase* activities in the presence and the absence of low salt washed ribosomes.

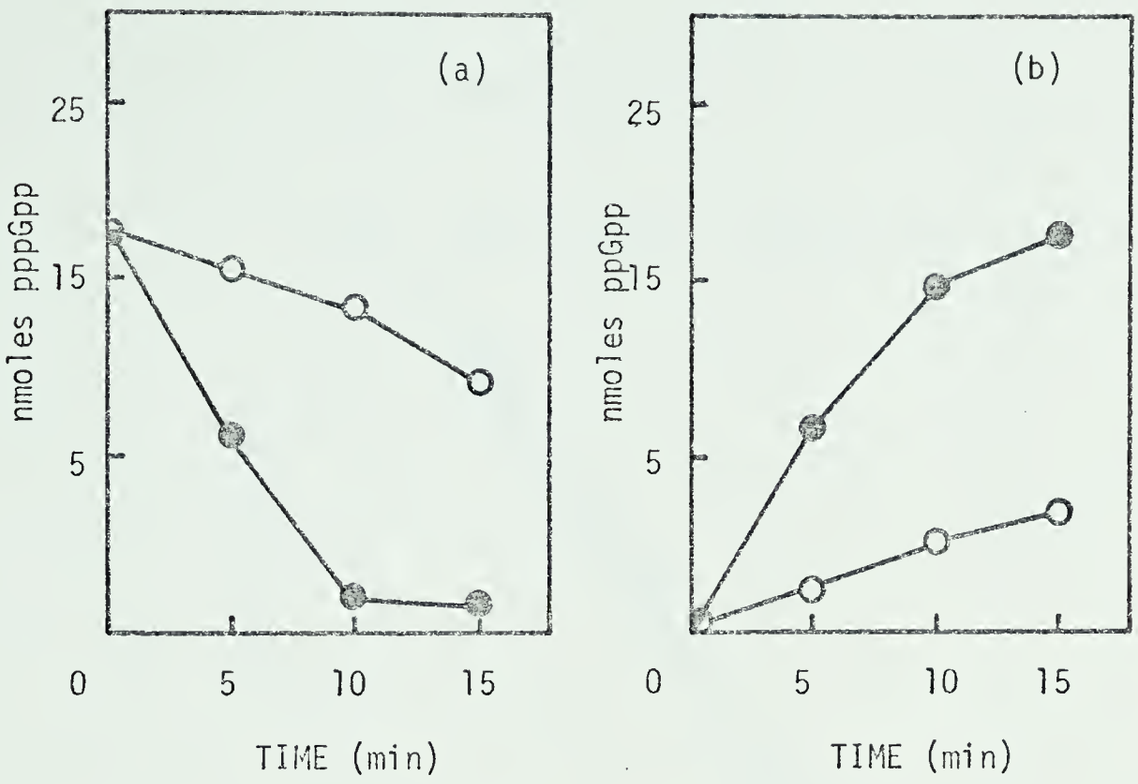

Figure 10 - Hydrolysis of pppGpp by S-30 extracts of a wild type strain and a *gpp* mutant.

The reaction mixture contained: 80 ul [^{32}P]pppGpp (22.3 nmoles; 1150 CPM / nmole) in water, and 60 ul of S-30 extract (1.8 mg/ml protein in buffer-A). The reaction mixtures were incubated at 32°. At the indicated intervals, 30 ul samples were withdrawn into 10 ul of 4 M formate, and chromatographed on PEI cellulose thin layers. (a) shows the rate of pppGpp hydrolysis, (b) shows the rate of ppGpp accumulation. Symbols: (o) MS240 (*gpp*$^-$), (●) CS251 (*gpp*$^+$).

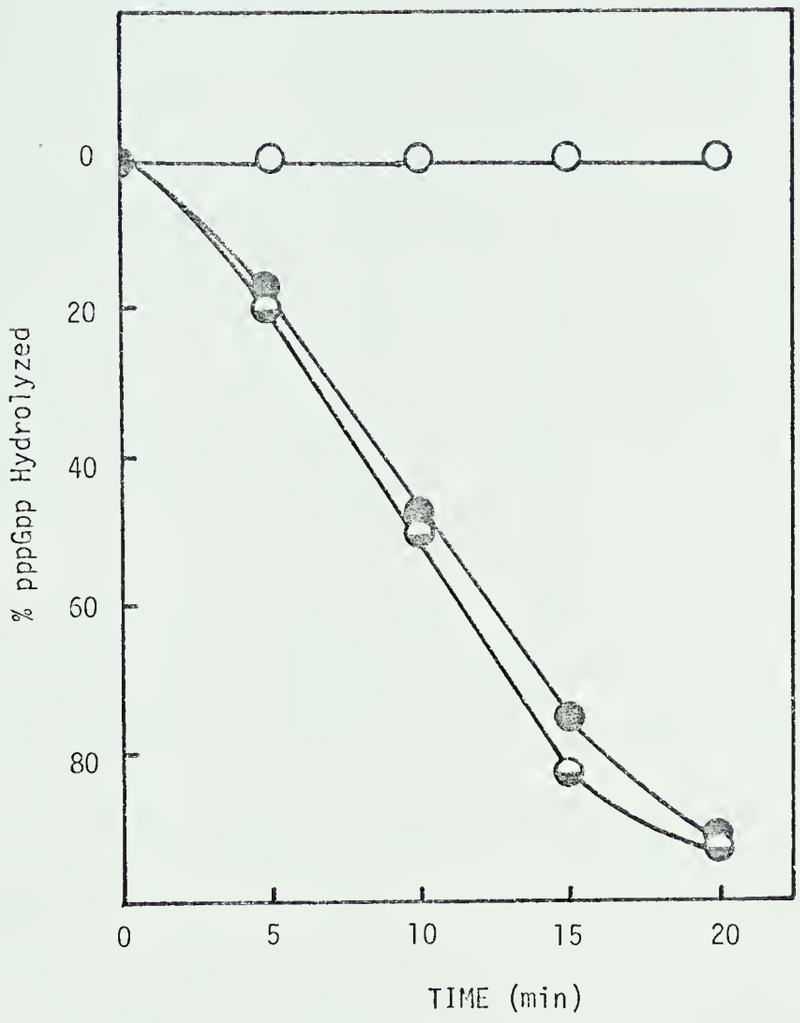

Figure 11 - Hydrolysis of pppGpp by S-100 extracts from various strains. The 320 ul reaction mixtures contained 200 ul of S-100 extract (6mg/ml of protein in buffer-A), and 80 ul of 3'-[α^{32}P]pppGpp (3.8 uCi/umole) in buffer-A. The final concentration of pppGpp was 0.25 mM. The reactions were incubated at 32°. At the indicated intervals, 50 ul samples were withdrawn into 10 ul of 5 M formate. Aliquots of the acidified reaction mixtures were chromatographed on PEI cellulose thin layers, and the areas of interest were cut out and counted. Symbols: (●) CS417 (*gpp*$^+$ *spoT*$^+$); (◐) CS412 (*gpp*$^+$ *spoT*$^-$); (○) CS416 (*gpp-1 spoT*$^+$); CS425 (*gpp-2 spoT*$^+$); and CS426 (*gpp-3 spoT*$^+$).

The results from the first series of assays, in which fractions of the wild type extract were assayed in the absence of ribosomes, are presented in Figure 12. From these results it appears that there are at least two major ribosome-independent pppGpp*ase* activities in the wild type extract. The relatively small shoulder of activity (peak II-A) was repeatedly observed (Appendix I) however, the relative level of activity varied substantially in four separate preparations. The first peak of activity is very labile under these conditions. The activity is almost completely degraded 24 hours after the preparation of the extract. The activity of this peak may exceed that of peak II if assayed as it emerges from the column, and the activity is not affected by the presence of ribosomes (Appendix I). This peak appears to contain a non-specific phosphatase since only 50% of the pppGpp hydrolyzed is recovered as ppGpp (Figure 12). This peak also hydrolyzes p-nitrophenyl-phosphate (a substrate for a variety of phosphatases), but does not appear to hydrolyze ppGpp.

When the wild type fractions were assayed in the presence of low salt washed ribosomes an additional two peaks of pppGpp*ase* activity were observed (Figure 13). These activities probably represent EF-G and EF-T which have previously been shown to catalyze the ribosome-dependent hydrolysis of pppGpp (Hamel and Cashel, 1973). As expected, these fractions also contain GTP*ase* activity (Appendix I). The absence of the "peak I" pppGpp*ase* is attributed to the degradation of this activity during the 12 hour interval between the assays presented in Figures 12 and 13.

Fractionation of the pppGpp*ase* activities of the *gpp-1* mutant extract revealed that the major ribosome-independent pppGpp*ase*

activity and the relatively smaller shoulder of activity were completely missing (Figure 14). The absence of *two* peaks of activity (peaks II-A and II-B; Figure 12) was unexpected, but might be explained by the assumption that the *gpp* enzyme is active in two aggregate forms. Since DEAE Sephadex separates on the basis of both size and charge, the observed pattern of separation of the two activities might result. Alternatively, it is possible that the *gpp* mutant carries mutations in two tightly linked genes of similar function. Since no readily observed phenotype has been ascribed to the *gpp* mutations, it has not been possible to exclude this possibility by the selection of revertants.

A relevant ancillary question concerns the specificity of the various pppGpp*ase* activities. Since EF-G and EF-T have been demonstrated to possess both GTP*ase* and pppGpp*ase* activities, it seemed possible that the peak II nucleotidase might also hydrolyze nucleotides other than pppGpp. This was examined by assessing the ability of several related nucleotides to act as competitive substrates. The ideal approach to this problem would have been to determine the Ki of the various nucleotides. However, in order to obtain accurate Km and Ki estimates, the assay must be sensitive enough to detect hydrolysis of even a few percent of the lowest concentration of substrate. Since the specific activity of the [^{32}P]pppGpp available at this time was not high enough to satisfy this criterion, a less critical assay was employed.

A competition assay was conducted by simply incubating the partially purified peak II-B pppGpp*ase* from the wild type extract with [^{32}P]pppGpp and another nucleotide, and measuring the amount of pppGpp

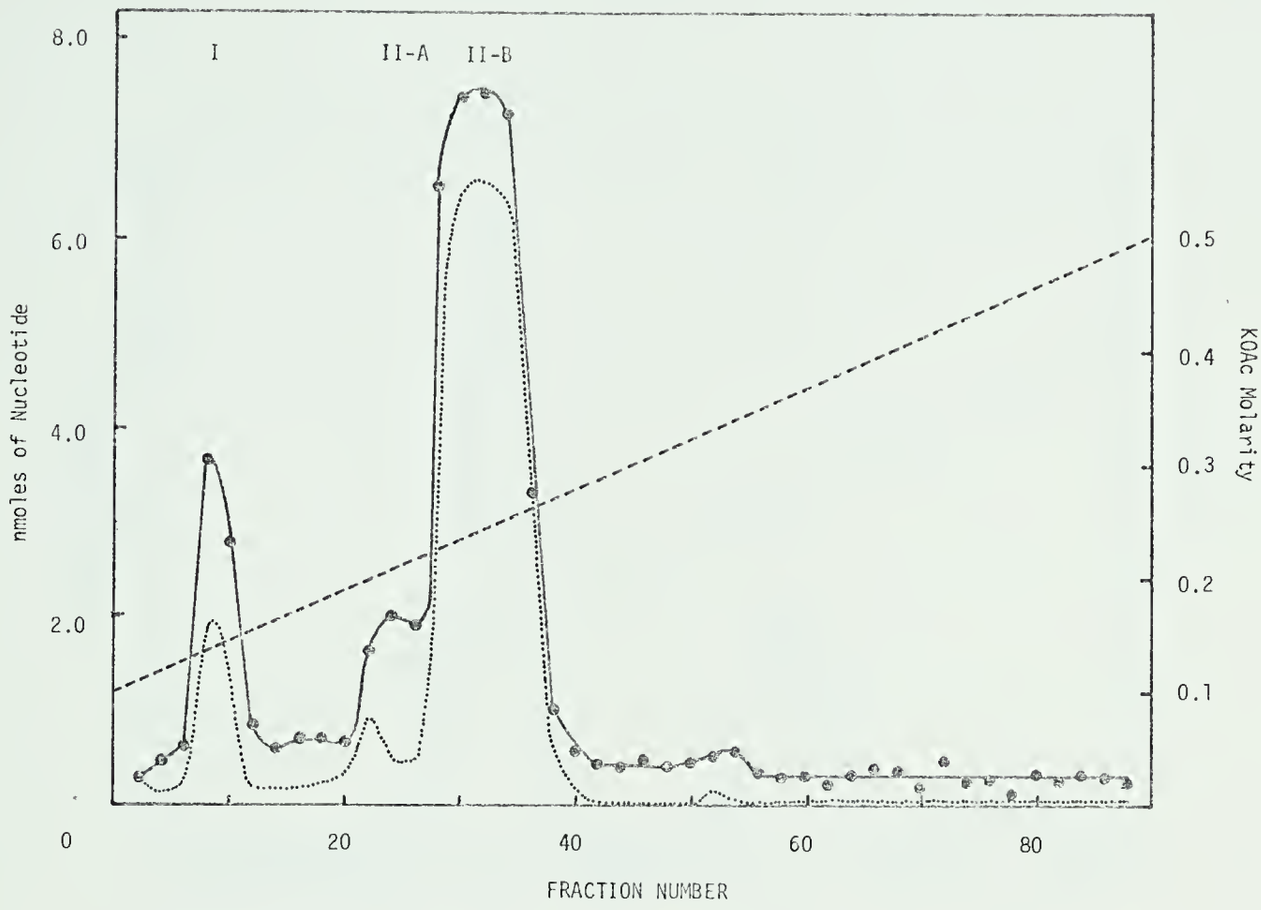

Figure 12 - Ribosome-independent pppGpp*ase* activities from a wild type strain
An S-100 extract of the wild type strain CS417 was chromatographed on DEAE Sephadex as described in *Materials and Methods*. The dialyzed fractions were assayed in 40 ul reactions containing 10 ul of 3'-[α^{32}P]pppGpp (7.7 nmoles), 20 ul of enzyme, and 10 ul of buffer-A. The reaction mixtures were incubated at 32° for 60 min, and the reaction was terminated by the addition of 5 ul of 5 M formate. A 20 ul aliquot of the reaction mixture was chromatographed on PEI cellulose, and the areas of interest were cut out and counted. The solid line represents the amount of pppGpp hydrolyzed, and the dotted line represents the amount of ppGpp produced.

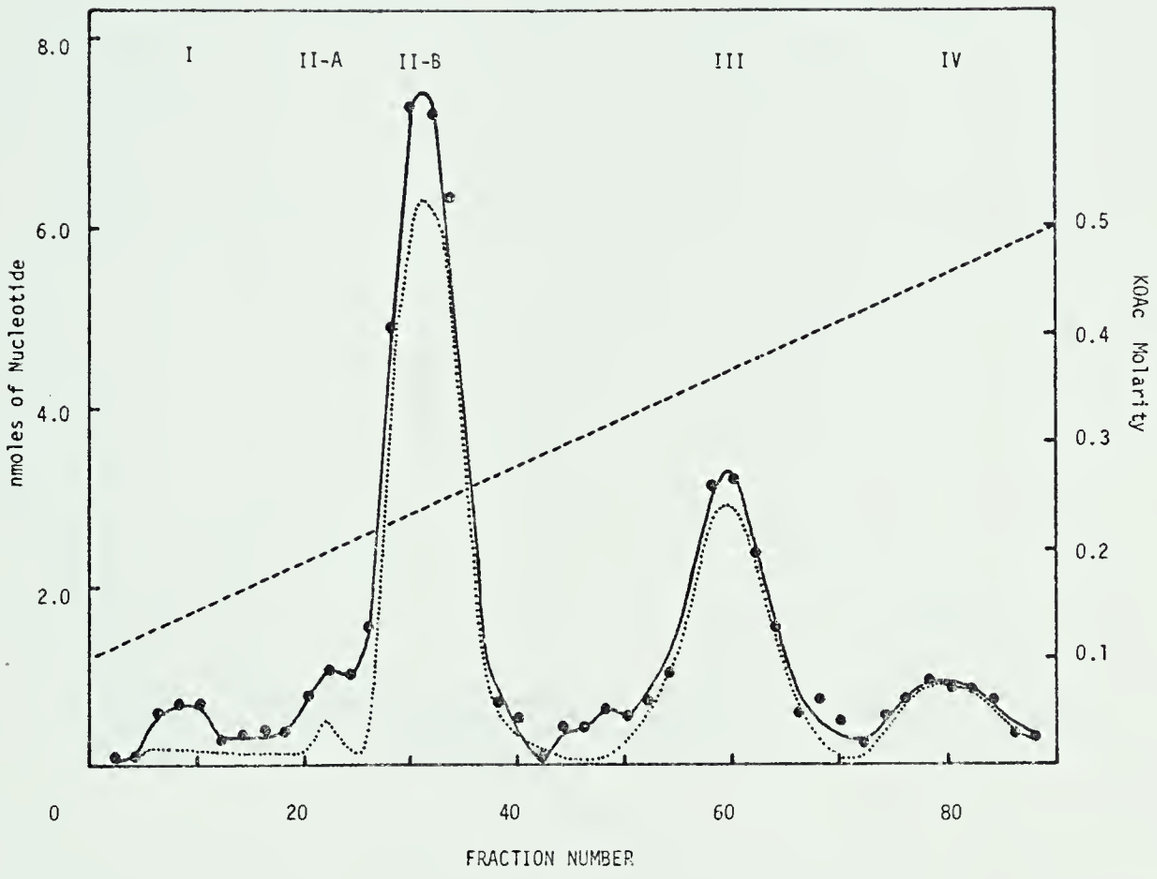

Figure 13 - The pppGpp*ase* activities from the wild type strain when assayed in the presence of ribosomes.

An S-100 extract of the wild type strain CS417 was prepared, fractionated, and assayed as described in Figure 12, except that 2.5 A_{260} units of low-salt washed ribosomes were included in the reaction mixture. The solid line represents pppGpp hydrolyzed, and the dotted line represents ppGpp produced.

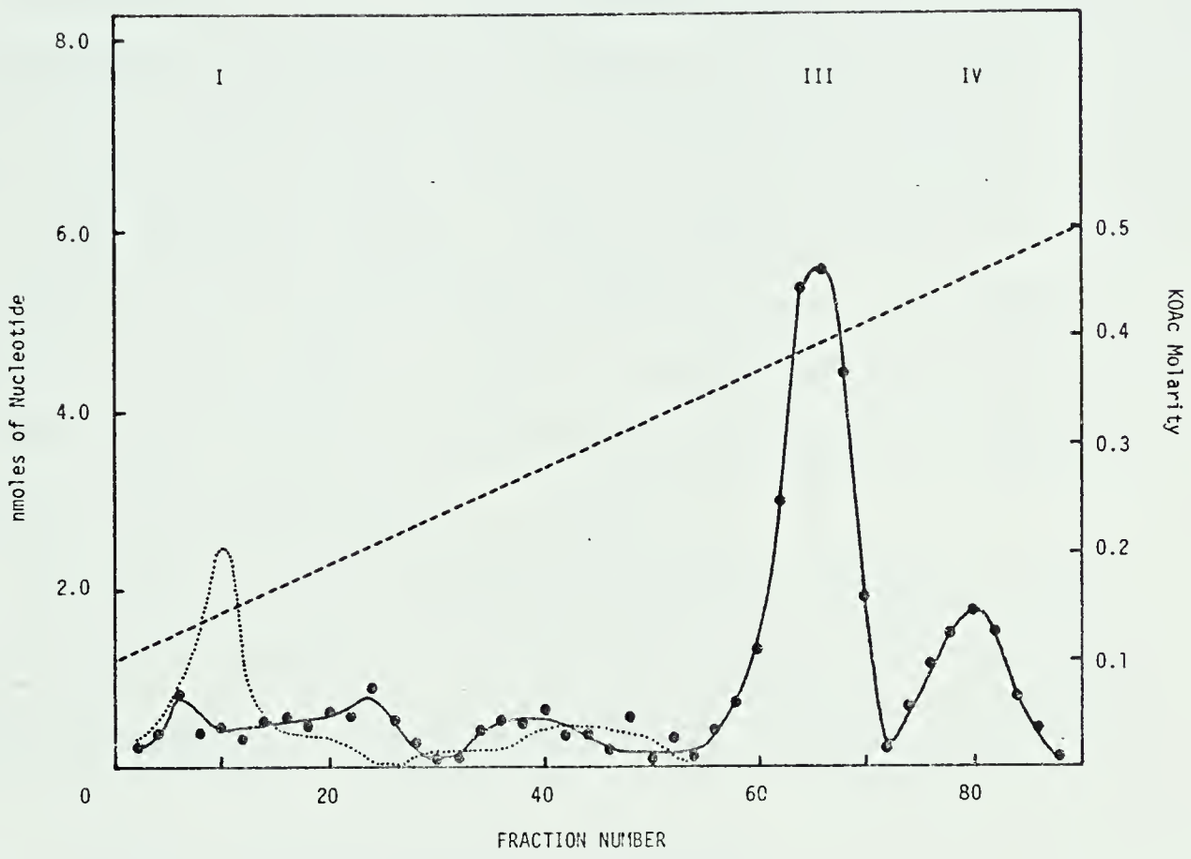

Figure 14 - Fractionation of pppGpp*ase* activities from a *gpp* mutant.
An S-100 extract of the *gpp* mutant CS416 was prepared, fractionated, and assayed as described in Figures 12 and 13. The solid line represents pppGpp hydrolyzed in the presence of ribosomes, and the dotted line represnts pppGpp hydrolyzed in a ribosome-free reaction.

hydrolysis. The results of this experiment, presented in Table 4, suggest that GDP, GTP, ATP and UTP, are not competitive inhibitors. It is, therefore, tentatively concluded that the enzyme(s) specified by the *gpp* gene, exhibits considerable substrate specificity for pppGpp.

The product of the *gpp*-enzyme mediated hydrolysis of pppGpp is not distinguishable from ppGpp by one dimensional chromatography in 1.5 M KH_2PO_4 on PEI cellulose thin layers. The hydrolysis product is, therefore, probably pppGp or ppGpp. Since the hydrolysis product is not a good substrate for 3'-nucleotidase (Appendix II), it is tentatively concluded that the product is ppGpp. A more definitive conclusion would have been obtained from the experiment presented in Appendix II if pppGp had been available as a control.

No attempt was made to determine optimal conditions for the assay of peak II pppGpp*ase*. It was noted however, that the enzyme activity disappears in four days when stored in buffer-A at 4°.

Effect of a relA mutation on the gpp phenotype

The effect of a *relA* mutation upon the *gpp* phenotype was investigated by constructing a series of isogenic strains, in which the two genes were combined in all possible combinations. The strains were constructed by first transducing the *gpp relA* strain CS421 (F^- *argH relA str spc xyl metE gpp-1 thi*) to arg^+ with a P1*vir* lysate of the strain CS305 (Hfr $relA^+$ *thi*). A rel^+ transductant from this cross was retained and designated CS422 (F^- *str spc xyl metE gpp-1 thi*). The strain CS422 was then transduced to met^+ with a P1*vir* lysate of CS305. A gpp^+ transductant from this cross was retained, and designated CS423 (F^- *str spc*

Table 4

The effect of various nucleotides on fraction II pppGpp*ase* activity.

Nucleotide added	pppGpp*ase*-II relative activity
0	1.00
GDP	0.99
GTP	0.97
ATP	0.94
UTP	0.98

The pppGpp*ase* activity was measured in 90 ul reaction mixtures containing 50 ul of wild type peak II pppGpp*ase* (3 ug of protein), 20 ul of 3'-[β-^{32}P]pppGpp (3.8 uCi/umole; 0.2 mM final concentration), and 20 ul of GDP, GTP, ATP or UTP at a final concentration of 1.0 mM. The final concentration of buffer and salts was the same as for buffer-A (*Materials and Methods*). The reaction was incubated at 32° for 30 min, and terminated by the addition of 10 ul of 5 M formate. Aliquots of the reaction mixture were chromatographed on PEI cellulose thin layers, and the areas of interest were cut out and counted. The substrate was present in excess, since less than 40% of the pppGpp was hydrolyzed during the course of the reaction. The pppGpp*ase* activity is presented relative to the control, which had a specific activity of 13.1 umoles pppGpp hydrolyzed per mg of protein in 30 min.

xyl thi). The final member of the series, a *gpp*$^+$ *relA*$^-$ derivative, was constructed by tranducing CS421 to met$^+$ with a P1*vir* lysate of CS305. Since the *relA* mutation prevents expression of the *gpp* phenotype, a number of randomly chosen transductants were screened for the presence of the *gpp* mutation by crossing out the *relA* mutation by performing a conjugal cross between Hfr CS305 and each of the tranductants. By this method a gpp$^+$ transductant, designated CS424 (F$^-$ *argA relA str spc xyl thi*), was identified.

The growth rates of the four related strains were found to be identical (57 min doubling time at 30° in Tris minimal supplemented with leucine, proline, histidine, arginine, methionine, and isoleucine), indicating that the various combinations of the *gpp* and *relA* mutations exert no deleterious effects upon the growth of the organism under these circumstances. As expected, the *relA* mutation is completely epistatic to the *gpp* mutation in that it prevents the accumulation of the MS-nucleotides following amino acid starvation (Figure 15).

Effect of the gpp mutation upon the downshift response

When rel$^+$ strains of *E. coli* are subjected to a decrease in the availability of a good carbon source, ppGpp accumulates at levels approaching those observed during amino acid starvation, but pppGpp does not accumulate. This well-documented, but poorly understood, response is generally believed to reflect a decrease in the turnover rate of ppGpp, rather than an increased rate of synthesis. According to this hypothesis, it might be expected that a mutation affecting the

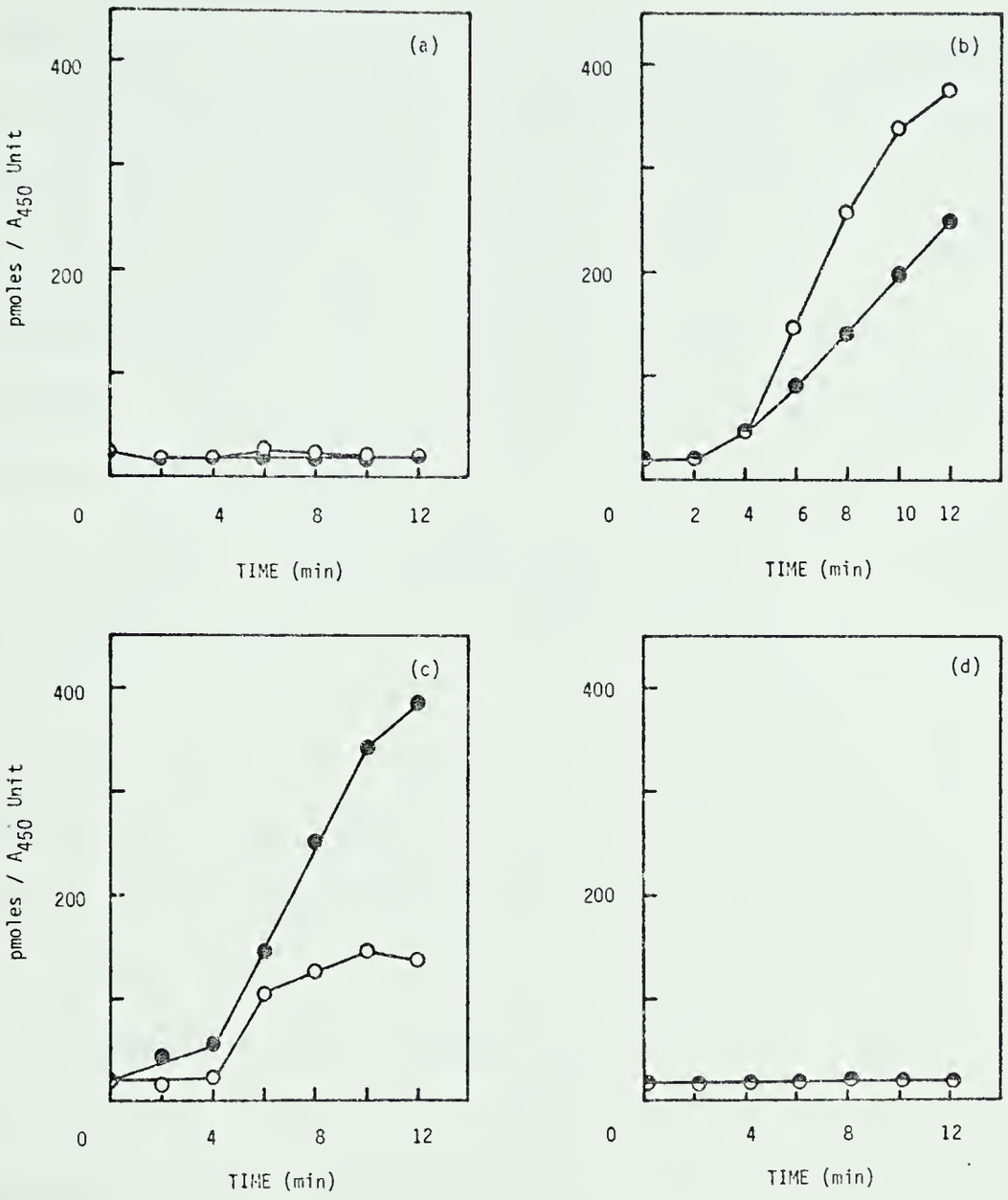

Figure 15 - The effect of various combinations of the *relA* and *gpp* mutations on the accumulation of the MS-nucleotides under conditions of amino acid starvation.

Cultures growing in Tris minimal were subjected at time 0 to serine hydroxamate induced serine starvation. Samples of the culture were withdrawn at the indicated intervals, and assayed for the levels of the MS-nucleotides as described in *Materials and Methods*. (a) CS421 (*relA*$^-$ *gpp*$^-$), (b) CS422 (*relA*$^+$ *gpp*$^-$), (c) CS423 (*relA*$^+$ *gpp*$^+$), and (d) CS424 (*relA*$^-$ *gpp*$^+$). Symbols: (•) ppGpp, (o) pppGpp.

turnover rate of pppGpp should not have any pronounced effect upon the level of the MS-nucleotides.

The effect of a *gpp* mutation upon the downshift-induced accumulation of the MS-nucleotides was examined by subjecting an isogenic pair of strains to α-methylglucoside induced downshift. From the results of this experiment, illustrated in Figure 16, it can be seen that the *gpp* mutation causes a dramatic increase in the level of pppGpp accumulation. In contrast, the levels of ATP and GTP respond almost identically in the mutant and the wild type strain (Figure 17).

The simplest interpretation of this effect is that a significant amount of pppGpp synthesis occurs during downshift, but is obscured in the wild type strain by the rapid rate of pppGpp hydrolysis catalyzed by the *gpp*-enzyme. As an elaboration of this scheme, it might be postulated that other enzymes, which are normally capable of pppGpp hydrolysis, are inhibited during downshift. For example, the *spoT* gene product appears to be less active during downshift.

The downshift-induced accumulation of pppGpp is also observed in other *gpp* mutants (Figure 18), suggesting that the same mutation is responsible for the increased levels of pppGpp observed during both amino acid and glucose starvation.

The interaction of gpp and spoT

Following amino acid starvation *spoT* mutants rapidly accumulate high levels of ppGpp without the concomitant accumulation of pppGpp. Upon the release of starvation, the ppGpp pool declines at a rate characteristic of the particular *spoT* allele (Fiil *et al.*, 1977). As

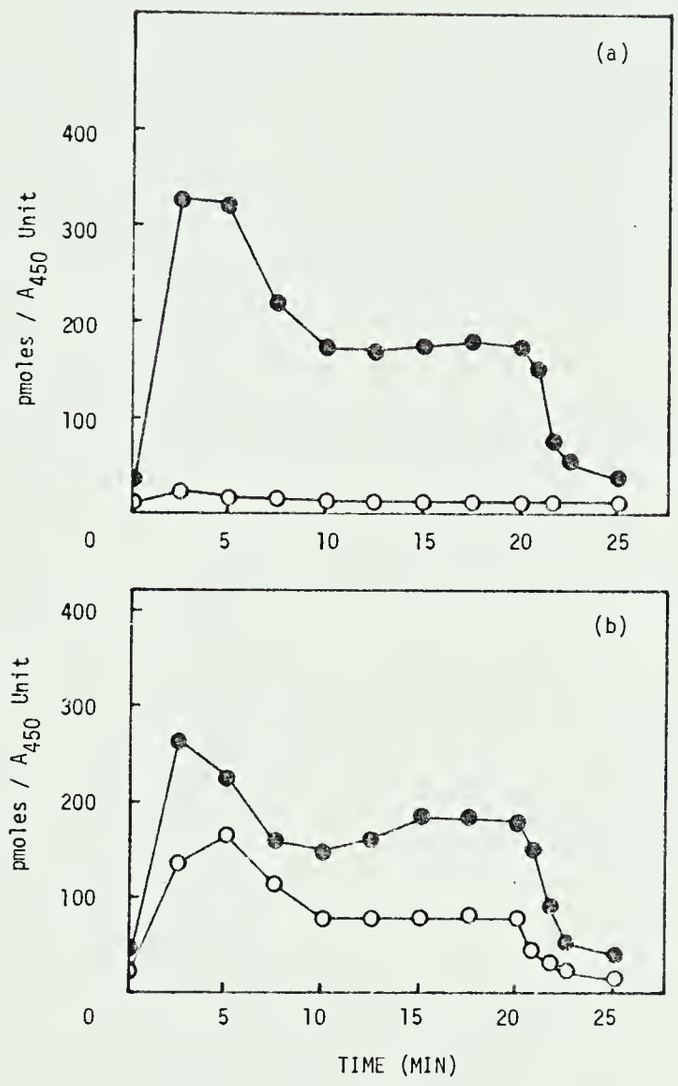

Figure 16 - Downshift induced accumulation of MS-nucleotides in the wild type and a *gpp* mutant.

Cultures were subjected to α-methylglucoside induced downshift, and the nucleotide pools were measured as described in *Materials and Methods*. At time 20, the downshift was reversed by the addition of glucose to 2%. (a) CS417 (gpp^+), and (b) CS416 (*gpp-1*). Symbols: (●) ppGpp, (○) pppGpp.

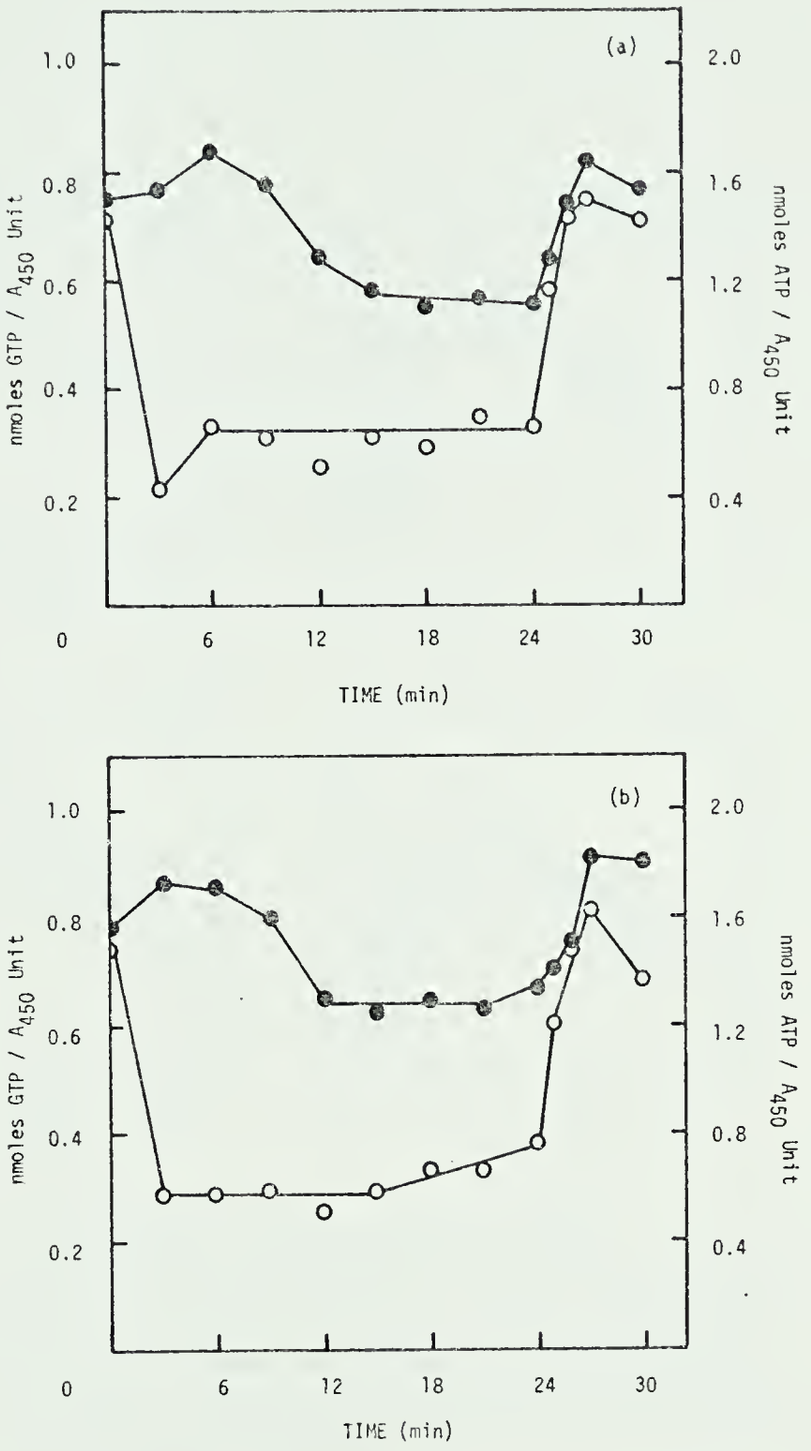

Figure 17 - Downshift induced changes in the ATP and GTP pools in the wild type and a *gpp* mutant.

The conditions of the assay are described in Figure 16. (a) CS417 (*gpp*$^+$), and (b) CS416 (*gpp-1*). Symbols: (•) ATP, (o) GTP.

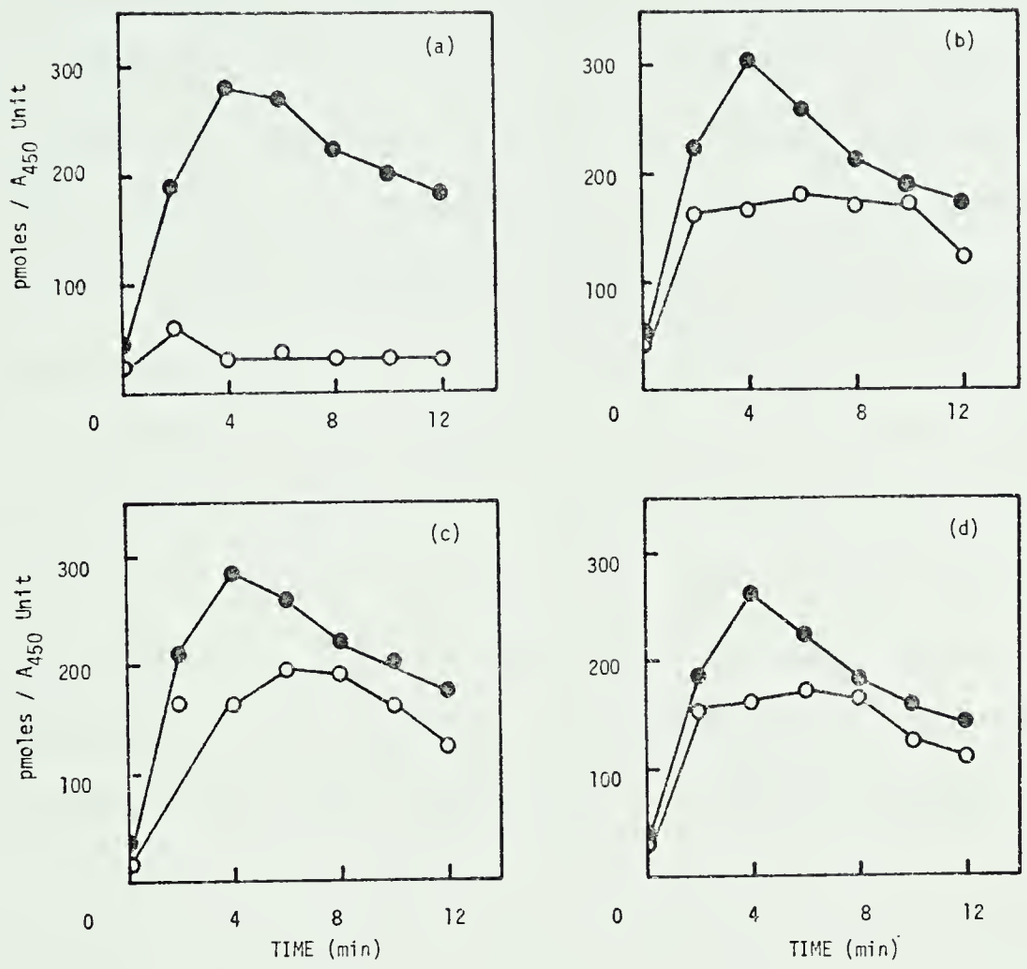

Figure 18 - Downshift induced accumulation of pppGpp in several *gpp* mutants.
Cultures growing in Tris minimal (0.1% glucose) were subjected to downshift by the addition, at time 0, of α-methylglucoside to a final concentration of 2%. Samples of the cultures were removed at the indicated intervals, and assayed for the levels of the nucleotide pools as described in *Materials and Methods*. (a) KL266 (*gpp*$^+$), (b) CS425 (*gpp-2*), (c) CS426 (*gpp-3*), and (d) CS427 (*gpp-4*). Symbols: (●) ppGpp, (○) pppGpp.

previously noted, these characteristics of the *spoT* mutants have been interpreted as evidence that *spoT* is involved in both the synthesis and degradation of ppGpp.

The pattern of (p)ppGpp accumulation in a *spoT* mutant undergoing amino acid starvation is similar to that observed in a *relA*$^+$ *spoT*$^+$ strain undergoing carbon source downshift. It was, therefore, expected that a *gpp spoT* mutant undergoing amino acid starvation would resemble a *gpp* mutant following carbon source downshift. However, several attempts to construct a *spoT gpp* strain failed to produce the double mutant. In the first attempt, a P1*vir* lysate of the *spoT* strain W2 (F$^-$ *spoT thr leu pro gal his arg thi*) was used to transduce the *gpp* mutant CS419 (F$^-$ *gpp pyrE leu ara pro lac his cys thy str spc thi*). Of 57 pyrE$^+$ transductants recovered from this cross, none were distinguishable from the *gpp*$^-$ *spoT*$^+$ recipient. Since the same lysate produced more than 30% *spoT*$^-$ transductants in a control experiment, it was concluded that either the *gpp* mutation prevented detection of the *spoT* mutation, or the double mutant was inviable. In order to examine the first possibility an attempt was made to introduce a *gpp* mutation into a *spoT* mutant. A P1*vir* lysate of the *gpp* strain CS403 (F$^-$ *gpp strA mtl glp met arg mal thi*) was used to transduce the *spoT* strain CS412 (F$^-$ *spoT pyrE metE leu ara his cys thy str spc thi*). The 43 metE$^+$ transductants recovered from this cross were indistinguishable from the *gpp*$^+$ *spoT*$^-$ recipient. In a previous experiment the cotransduction frequency for *metE* and *gpp* was 32% (Table 2). It was, therefore, tentatively concluded that the *spoT gpp* phenotype is inviable.

Additional evidence for the inviability of the *spoT gpp* combination was obtained by demonstrating a reduction in the cotransduction

frequency of outside markers. A P1*vir* lysate of the *gpp* strain CS415 (F$^-$ *gpp ilvD his cys thy str spc xyl thi*) was used to transduce the *spoT* strain CS412 (F$^-$ *spoT pyrE metE leu ara his cys thy str spc thi*). Of the 150 metE$^+$ transductants recovered from this cross, 5 were ilv$^-$. In the control experiment in which CS417 (F$^-$ *metE his cys thy str spc xyl thi*) was transduced to metE$^+$ with the same lysate of CS415, 35% of the transductants were ilv$^-$. Thus, the presence of the *gpp* mutation on the donor chromosome resulted in a substantial reduction in the co-transduction frequency of the outside markers when the recipient was *spoT*$^-$ but not when the recipient was *spoT*$^+$. The 5 *ilv*$^-$ transductants recovered from the CS415 x CS412 cross had a phenotype which was not distinguishable from the *spoT* recipient strain CS412. In order to ensure that the *spoT* mutation was not masking the effect of a *gpp* mutation, each of the 5 exceptional *ilv*$^-$ transductants was transduced to spoT$^+$ with a P1*vir* lysate of CS403 (F$^-$ *gpp str mtl ilv glp met arg mal thi*). All of the 5 transductants were clearly gpp$^+$. Thus, these 5 transductants probably received the *ilvD* mutation by a double cross-over event.

In order to examine the basis for the apparent inviability of the *gpp spoT* combination, an attempt was made to isolate a conditional mutation at one of the loci. An additional 6 *gpp* mutants were isolated by the procedures described previously, bringing the total number of *gpp* mutants to 15. However, none of these mutants exhibited a temperature conditional phenotype. Fortunately, a number of mutants, resembling *spoT* mutants, were incidentally recovered during the course of this study. The strain MS192, which was obtained from CS251 (F$^-$ *lac metG his thy str xyl mtl arg rif*^{r} *glp nal*) by nitrosoguanidine mutagenesis,

is identical to wild type with respect to (p)ppGpp accumulation at 32°, but *spoT* at 43.5°. The mutation responsible for this effect was transduced from the original mutant strain MS192 to CS411 (F^- *pyrE metE leu ara pro lac his cys str spc thi*) by selecting $pyrE^+$ transductants. Only a few transductants from this cross were scored so the precise map position of this mutation is not known. However, the similarity of map position and phenotype to that of the *spoT* mutants suggests that it is an allele of *spoT*. A transductant from the MS192 x CS411 cross was retained and designated CS444 (F^- $spoT^{ts}$ *metE leu ara pro lac his cys str spc thi*).

The pattern of (p)ppGpp accumulation during amino acid starvation of the $spoT^{ts}$ strain CS444 and the appropriate controls is presented in Figures 19 - 21. At 32° the pattern of (p)ppGpp accumulation in this strain is indistinguishable from that observed in the otherwise isogenic wild type strain CS411 (Figure 19). However, when a culture of CS444 is shifted to 43.5°, the $spoT^{ts}$ strain(s) rapidly accumulates ppGpp to a level which is 50% of the maximal level of ppGpp accumulation during amino acid starvation at 32° (Figure 20). Amino acid starvation at 43.5° results in a rapid increase in the already elevated level of ppGpp but does not provoke pppGpp accumulation. In this respect the strain behaves as a typical *spoT* mutant. However, when the starvation is alleviated, the level of ppGpp falls very rapidly to the prestarvation level. It, therefore, appears that the $spoT^{ts}$ mutation does not cause a significant reduction in the rate of ppGpp degradation. In view of this observation it seems necessary to propose that the increase in the level of ppGpp following the shift to 43.5° reflects either an increased

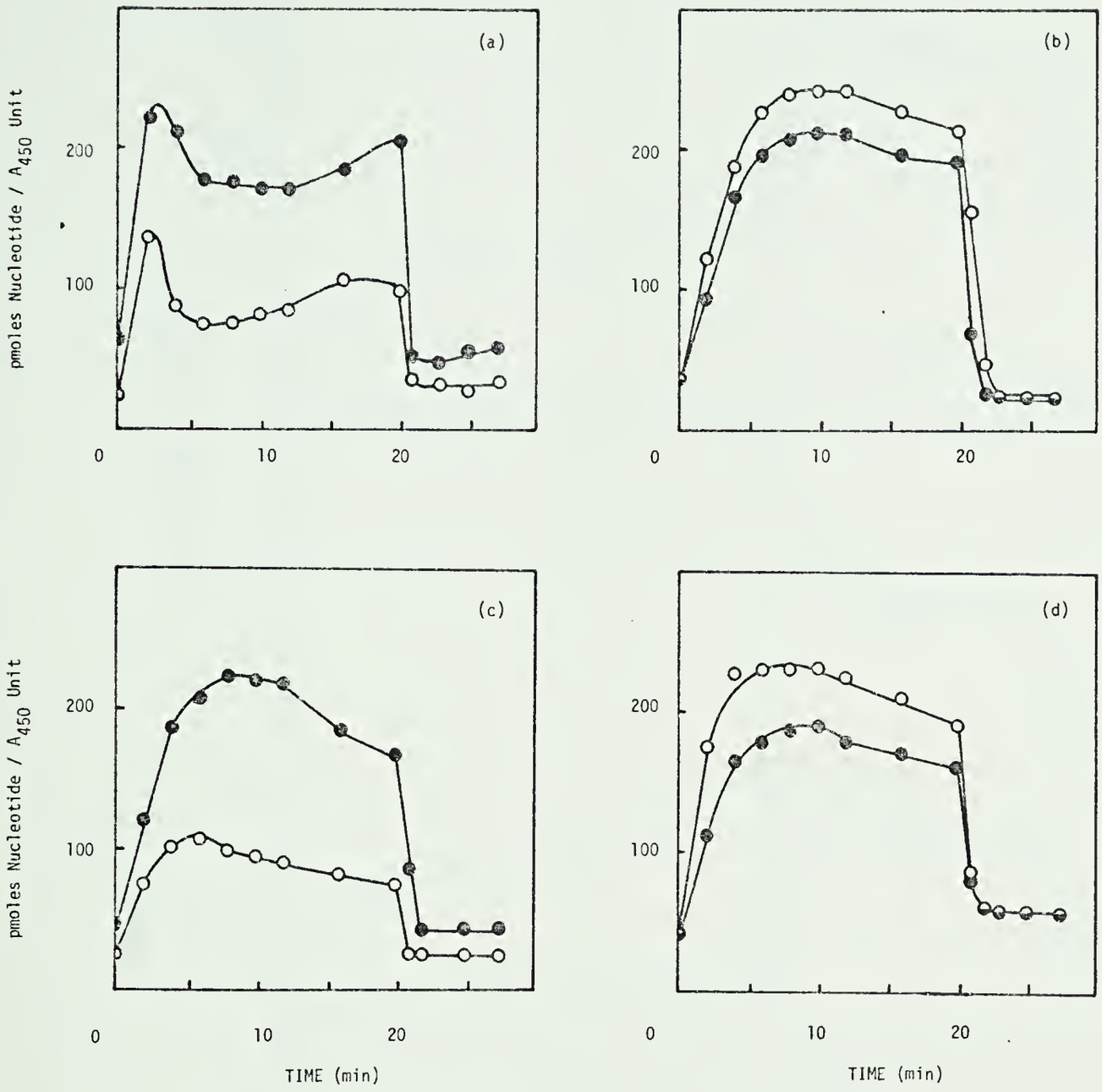

Figure 19 - MS-nucleotide accumulation in *spoT*^{ts} strains at 32°.

Cells grown in Tris minimal at 32° were subjected to isoleucine starvation at time 0. At the indicated intervals, samples of the culture were assayed for the levels of ppGpp and pppGpp. At 20 min, starvation was alleviated by the addition of L-isoleucine to a final concentration of 100 ug/ml. (a) CS411 (*gpp*$^{+}$ *spoT*$^{+}$), (b) CS416 (*gpp*$^{-}$ *spoT*$^{+}$), (c) CS444 (*gpp*$^{+}$ *spoT*^{ts}), and (d) CS445 (*gpp*$^{-}$ *spoT*^{ts}). Symbols: (•) ppGpp, (o) pppGpp.

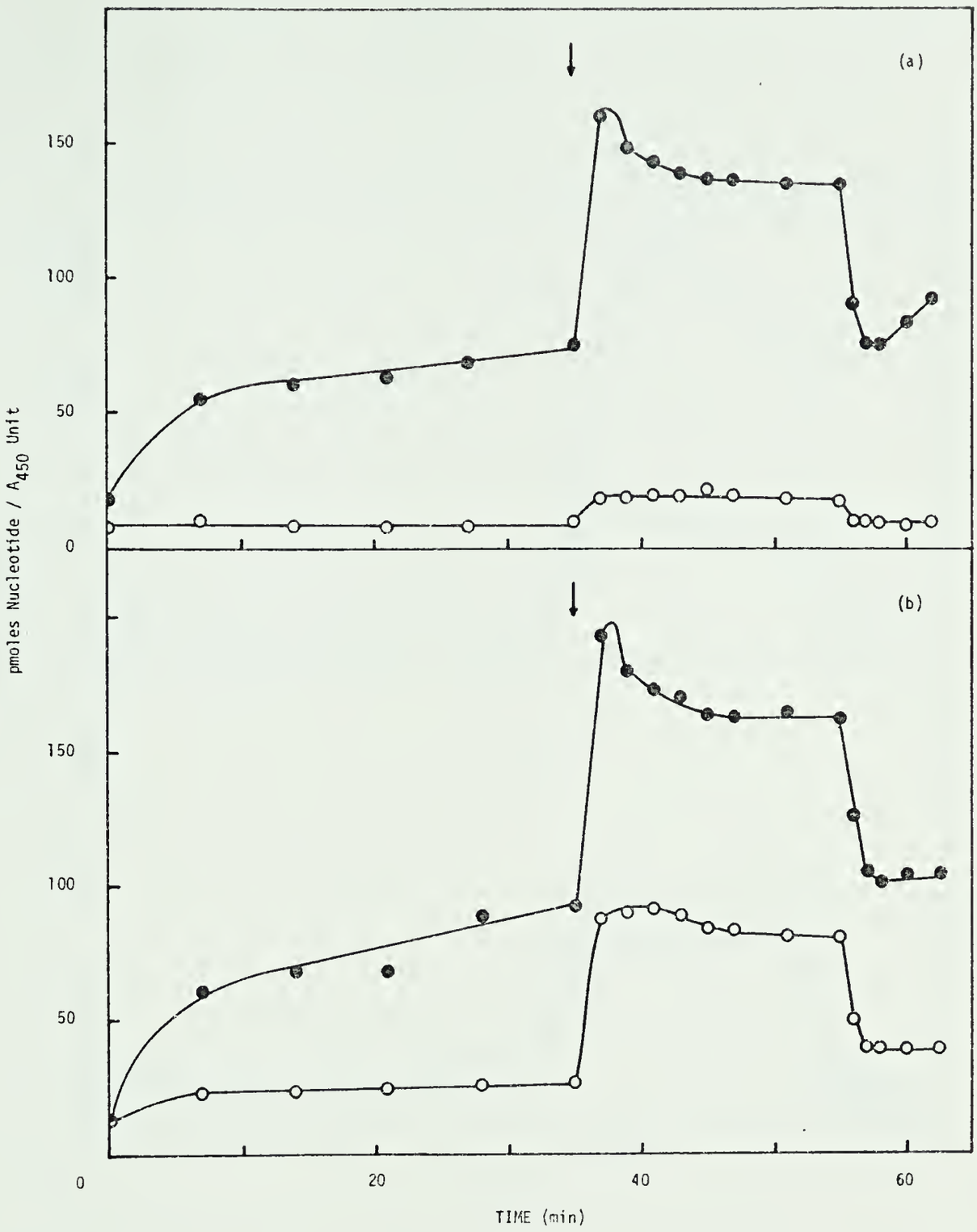

Figure 20 - MS-nucleotide accumulation in *spoT*^{ts} strains at 43.5°.

Cells grown in Tris minimal at 32° were shifted to 43.5° at time 0. At 35 min, isoleucine starvation was imposed (indicated by the arrow). At 55 min amino acid starvation was alleviated by the addition of L-isoleucine. At the indicated intervals, samples of the cultures were assayed for the levels of ppGpp and pppGpp. (a) CS444 (*gpp*$^{+}$ *spoT*^{ts}), and (b) CS445 (*gpp*$^{-}$ *spoT*^{ts}). Symbols: (•)ppGpp, (o) pppGpp.

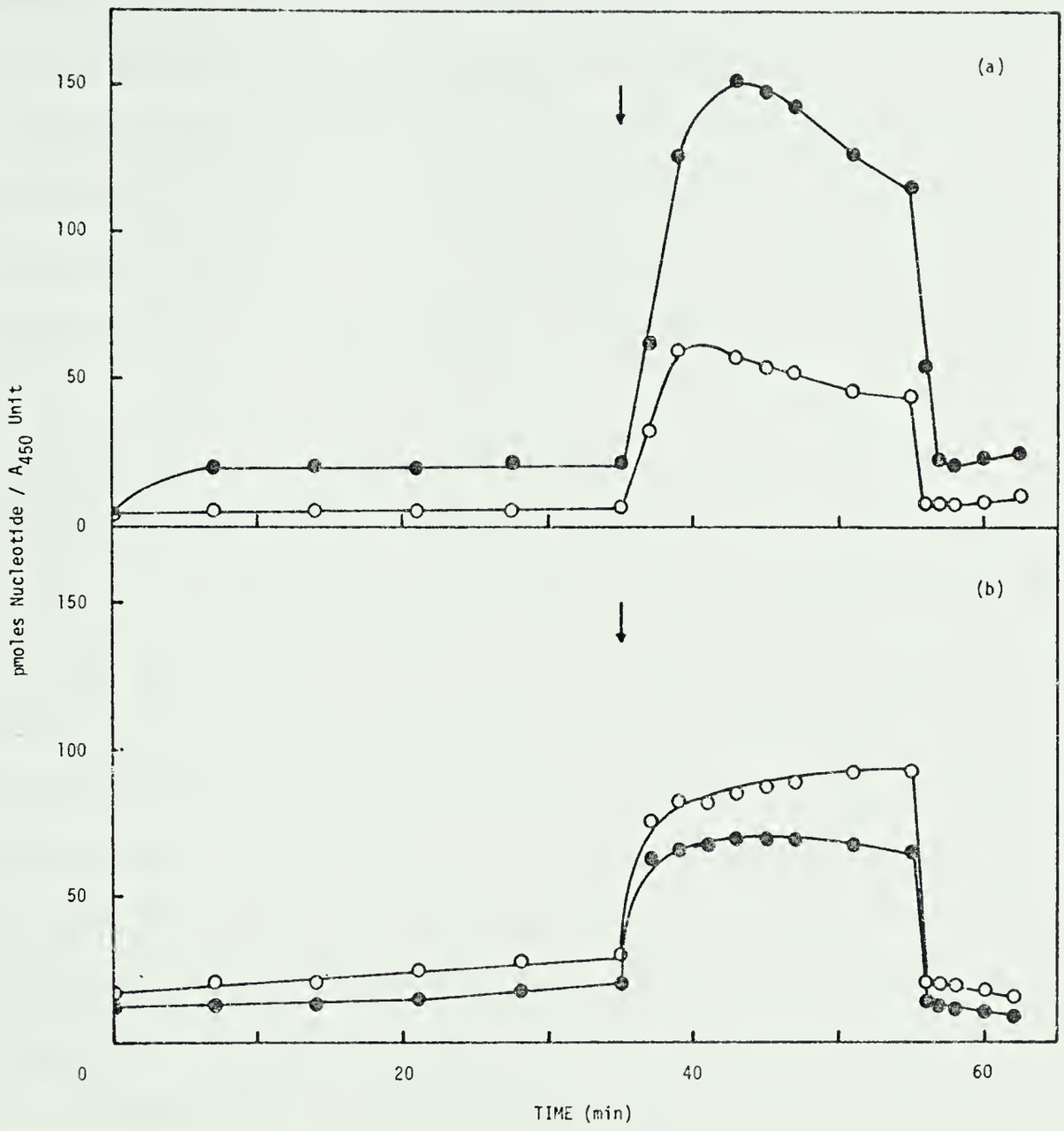

Figure 21 - MS-nucleotide accumulation in a *gpp* mutant and a wild type strain at 43.5°. The procedures were as described in Figure 20. (a) CS411 (*gpp*$^+$ *spoT*$^+$), and (b) CS416 (*gpp*$^-$ *spoT*$^+$). Symbols: (•) ppGpp, (o) pppGpp.

rate pf ppGpp synthesis, or a slight decrease in the rate of ppGpp degradation without any decrease in the rate of synthesis. These alternatives do not appear sufficient to explain the low level of pppGpp accumulation during amino acid starvation of the *spoT*^{ts}.

Although the *spoT*^{ts} mutation is anomalous in several respects, it was nonetheless of interest to determine the effect of a *gpp* mutation upon the *spoT*^{ts} phenotype. The double mutant was constructed by transducing the *spoT*^{ts} strain CS444 (F$^-$ *spoT*^{ts} *metE leu ara pro lac his cys str spc thi*) to metE$^+$ with a P1*vir* lysate of the *gpp* strain CS403 (F$^-$ *gpp-1 strA mtl glp met arg mal thi*). A *gpp spoT*^{ts} strain was recovered from this cross and designated CS445 (F$^-$ *gpp spoT*^{ts} *leu ara pro lac his cys str spc thi*). At 32° this strain is not distinguishable from the *gpp spoT*$^+$ strain CS416 (F$^-$ *gpp-1 his cys thy str spc xyl metE*) (Figure 19). The only major difference is that the *gpp spoT*^{ts} strain accumulates essentially wild type levels of pppGpp. The *gpp* mutation, therefore, effectively suppresses one effect of the *spoT*^{ts} mutation. As previously noted, this effect was expected by analogy with the results obtained during downshift of *gpp* mutants. This result is interpreted as evidence that substantial pppGpp synthesis occurs in the *spoT*^{ts} strains following amino acid starvation at 43.5°. The low level of pppGpp accumulation in the *spoT*^{ts} *gpp*$^+$ strain must, therefore, be due to an increased rate of pppGpp degradation or to a complex interaction between the *spoT* and *gpp* products. The latter possibility is discussed in detail under *Discussion*.

As previously noted, certain combinations of the *spoT* and *gpp* alleles appear to be inviable. A similar effect is observed in the

spoT^{ts} *gpp* mutant CS445 at 46°. At this temperature, which is extreme for *E. coli*, the wild type strain CS411 and the *gpp* mutant CS416 exhibit good growth in minimal medium supplemented with the required amino acids and 1.0% casamino acids. The *spoT*^{ts} strain CS444, which has a slower growth rate than the wild type at all temperatures, also grows at 46°. The *spoT*^{ts} *gpp* strain CS445 fails to grow on casamino acids or L-broth at 46°. A number of spontaneous temperature resistant mutants of CS445 have been selected by plating cells for growth at this temperature; however, these have not been characterized.

Since there was a detectable difference in the growth of the *spoT*^{ts} strain and the otherwise isogenic *spoT*^{ts} *gpp* strain at 46°, a preliminary investigation of the pattern of RNA synthesis at this temperature was undertaken. Cells growing at 32° in Tris minimal medium supplemented with casamino acids were shifted to 46° and stable RNA synthesis was monitored by measuring the accumulation of labeled uracil into TCA-precipitable material. The results of this experiment, presented in Figure 22, demonstrate a dramatic difference between the *spoT*^{ts} strain CS444 and the *spoT*^{ts} *gpp* strain CS445. Three minutes after the temperature shift, RNA accumulation (presumably synthesis) has ceased in the *spoT*^{ts} *gpp*$^{-}$ strain but not in the *spoT*^{ts} *gpp*$^{+}$ strain. It is, therefore, apparent that the direct or indirect products of the *gpp* and *spoT* genes interact in some manner.

There does not appear to be any simple scheme to account for the differential rate of RNA synthesis in the *spoT*^{ts} strain and the *spoT*^{ts} *gpp* strain. However, several points are worth noting. From the results presented in Figure 22 it can be seen that even at the permissive temperature, the strains carrying the *spoT*^{ts} mutation have a 3- to 4-fold

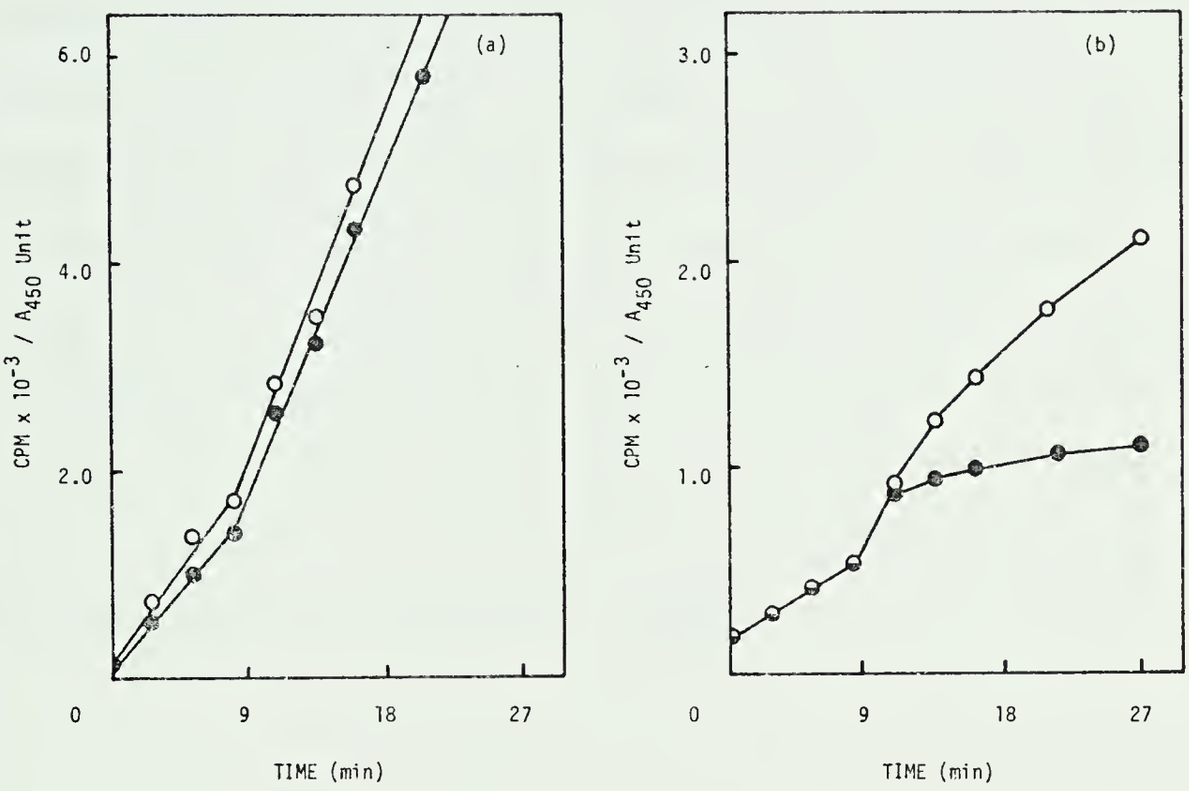

Figure 22 - RNA accumulation in *spoT*^{ts} and *gpp* mutants following a temperature shift.
Cells were grown at 32° in Tris minimal supplemented with 1.0% casamino acids and 10 ug/ml uracil. At time 0, the culture was supplemented with [^{3}H]-uracil to a final specific activity of 83 uCi/umole. At 9 min, the culture was shifted to 46°. At the indicated intervals, 120 ul samples of the culture were added to 3 ml of 5% TCA (0°). The precipitate, collected by filtration on GF/C filters, was washed 3 times with 3 ml of 5% TCA (0°), dried and counted. (a): (•) CS411 (*gpp*$^{+}$ *spoT*$^{+}$), (o) CS416 (*gpp*$^{-}$ *spoT*$^{+}$); (b): (•) CS445 (*gpp*$^{-}$ *spoT*^{ts}), (o) CS444 (*gpp*$^{+}$ *spoT*^{ts}).

lower rate of RNA accumulation. Since the pattern of (p)ppGpp synthesis is normal at this temperature it appears that this effect cannot be directly attributed to (p)ppGpp. Furthermore, since the prestarvation pattern of (p)ppGpp accumulation at 43.5° does not differ between the *spoT*^{ts} mutant and the *spoT*^{ts} *gpp* mutant (Figure 20), it appears that the difference in RNA accumulation at elevated temperature cannot be directly attributed to the level of (p)ppGpp. This raises the possibility that the *gpp* and *spoT* gene products both participate in a process other than the hydrolysis of pppGpp and ppGpp respectively. This proposal is consistent with the observation that *spoT* mutants exhibit a reduced rate of (p)ppGpp synthesis during amino acid starvation (Fiil *et al.*, 1977). Since this property of *spoT* mutants is not apparent *in vitro* (De Boer *et al.*, 1977), it seems possible that the *gpp* and *spoT* gene products are involved in a regulatory capacity.

DISCUSSION

There are three hypotheses concerning the precursor-product relationship of ppGpp and pppGpp: (i) ppGpp is the precursor of pppGpp; (ii) pppGpp is the precursor of ppGpp; and (iii) ppGpp and pppGpp are synthesized independently from GDP and GTP, respectively. The different approaches which have been employed in an attempt to verify these schemes are considered below.

In vitro experiments

The ribosome-mediated *in vitro* synthesis of ppGpp and pppGpp requires the product of the *relA* gene (stringent factor), the 30S and 50S ribosomal subunits, mRNA, and an uncharged tRNA capable of recognizing a codon carried by the message (Haseltine and Block, 1973; Pedersen *et al.*, 1973). When GDP is the substrate, ppGpp is the only product of the reaction. However, when GTP is the substrate both ppGpp and pppGpp are produced. The ratio of ppGpp to pppGpp decreases when more highly purified ribosomes are used to catalyze the reaction. It is, therefore, inferred that the accumulation of ppGpp is due to the presence of beta-gamma GTP*ases* which cosediment with the ribosomes and cause the hydrolysis of GTP to GDP, and pppGpp to ppGpp. EF-G, which is particularly difficult to remove from ribosomes, has been demonstrated to possess such hydrolytic activity (Hamel and Cashel, 1973; Cochran and Byrne, 1974). In the non-ribosomal reaction catalyzed by purified stringent factor, the product is exclusively pppGpp when GTP is used as the substrate, and ppGpp when GDP is the substrate (Block and Haseltine, 1974).

These results imply that, *in vivo*, ppGpp can be formed by the hydrolysis of pppGpp, or by stringent factor-mediated pyrophosphorylation

of GDP. Since the intracellular concentration of GDP is only 5% of the GTP level (Kari *et al.*, 1977), and since GDP and GTP are equally acceptable substrates for the stringent factor mediated reaction (Cochran and Byrne, 1974; Sy, 1974), it would be expected that the rate of direct synthesis of ppGpp from GDP should be only a small fraction of the rate of pppGpp synthesis. This expectation is based on the assumption that the precursors of (p)ppGpp are obtained directly from the free nucleotide pool. However, since several of the translation factors are GTP*ases*, it is possible that GDP, produced on the ribosome by a translation factor, is captured as a substrate by the stringent factor before it can be released from the ribosome. This proposal is entirely consistent with the results of the *in vitro* ribosome-mediated reaction, in which, even though the GTP concentration vastly exceeds the GDP concentration, the major product is ppGpp. Thus, it appears that the *in vitro* evidence does not discriminate between a model in which pppGpp is the major precursor of ppGpp, and one in which the two nucleotides are synthesized independently. There is no evidence from *in vitro* studies which might implicate ppGpp as a precursor of pppGpp.

In vivo experiments

The results of *in vivo* experiments, in which the kinetics of (p)ppGpp synthesis and decay were examined in detail, have been interpreted as evidence that the majority of ppGpp is derived by hydrolysis of pppGpp. When wild type cells, undergoing amino acid starvation, are supplemented with [^{3}H]-guanosine, the specific activity of the nucleotide pools increase in the order GTP, pppGpp, ppGpp (Chaloner-Larsson and

Yamazaki, 1976; Fiil *et al.*, 1977). These results are clearly inconsistent with a scheme in which ppGpp is the precursor of pppGpp, but favor the converse. These results also argue against the independent synthesis of ppGpp and pppGpp. Since GDP is the precursor of GTP, the specific activity of the GDP pool should increase more rapidly than that of the GTP pool. Thus, if GDP from the free nucleotide pool was a major precursor of ppGpp, the specific activity of the ppGpp pool would be expected to increase more rapidly than that of the pppGpp pool.

Similar conclusions have been reached by comparing the rates of ppGpp and pppGpp decay, following the release of starvation conditions or the inhibition of (p)ppGpp synthesis by chloramphenicol (Kari *et al.*, 1977; Weyer *et al.*, 1977). It was observed that, under these conditions, an immediate and rapid decrease in the pppGpp pool precedes a similar, but slightly less rapid, decline of the ppGpp pool. The GDP and GTP pools simultaneously undergo transient increases, presumably due to the recycling of the 5'-guanosine polyphosphate moiety of (p)ppGpp. The absence of a transient increase in the pppGpp pool, during the degradation of ppGpp, is considered as evidence that ppGpp is not a precursor of pppGpp. Furthermore, a calculation of the absolute rates of (p)ppGpp synthesis and decay revealed that the rate of pppGpp decay is approximately equal to the rate of ppGpp synthesis, but that the converse is not true (Fiil *et al.*, 1977). These results, therefore, argue against a scheme in which ppGpp is the precursor of pppGpp, but do not eliminate the possibility that the two nucleotides are synthesized independently. The *in vivo* results are, therefore, suggestive but not compelling.

Genetic experiments

Genetic evidence for a precursor-product relationship between pppGpp and ppGpp is derived largely from analysis of *spoT* mutants. Following amino acid starvation, these mutants accumulate greater than wild type amounts of ppGpp, but fail to accumulate pppGpp (Laffler and Gallant, 1974; Stamminger and Lazzarini, 1974). *spoT* mutants also have a decreased rate of ppGpp degradation. Therefore, these are considered to be defective in an enzyme which is responsible for converting ppGpp to some unidentified compound. These characteristics of the *spoT* mutants were initially interpreted as evidence that the *spoT* gene product was a kinase, responsible for the phosphorylation of ppGpp to pppGpp. However, it has recently been demonstrated that, at the onset of amino acid starvation, *spoT* mutants show a rapid burst of synthesis of pppGpp, the concentration of which may transiently exceed that of ppGpp (Chaloner-Larsson and Yamazaki, 1976). The pppGpp level subsequently undergoes a rapid decline to a barely detectable level. This result, which indicates that *spoT* mutants are competent to synthesize pppGpp, eliminates the rationale for proposing ppGpp as a precursor of pppGpp.

In order to satisfy the requirements of a model in which pppGpp is the precursor of ppGpp, it is necessary to propose that the *spoT* mutation causes a reduction in the rate of pppGpp synthesis, but does not affect the rate of degradation. The cell would, therefore, possess excessive pppGpp hydrolytic activity, and pppGpp would not accumulate. In contrast, the decreased rate of ppGpp turnover, effected by the *spoT* mutation, would compensate for the reduced rate of ppGpp synthesis, and ppGpp would be expected to accumulate. This model has received support from several

authors who have reported that, in *spoT* mutants, the rate of pppGpp synthesis is reduced by as much as an order of magnitude during amino acid starvation (Fiil *et al.*, 1977; De Boer *et al.*, 1977). The results obtained with *spoT* mutants do not exclude the possibility of independent synthesis of the MS-nucleotides. The requirements of this model are satisfied by assuming that the *spoT* mutation causes an equivalent reduction in both ppGpp and pppGpp synthesis. Since the *spoT* mutants are also defective in ppGpp (but not pppGpp) degradation, ppGpp would accumulate but pppGpp would not.

Considered as a whole, the available evidence provides some positive support for the hypothesis that pppGpp is a major precursor of ppGpp. However, none of the results exclude the possibility of independent synthesis. A prerequisite of either model is the existence of one or more enzymes which are capable of causing the rapid degradation of pppGpp. It would be expected that a mutation in such an enzyme should distinguish between the models, by causing a reduction in the ppGpp pool in one case, but not in the other case.

On the basis of the foregoing considerations, the present study was undertaken with the objective of identifying mutants defective in the degradation of pppGpp. Several mutants, which accumulate greater than wild type levels of pppGpp during amino acid or carbon source starvation, were isolated and characterized. All of these mutants appear to have an alteration in a previously undescribed gene, which is closely linked to the *ilv* operon. This gene, tentatively designated as the *gpp* locus, appears to code for a nuclease which specifically hydrolyzes pppGpp to ppGpp. The most convincing evidence for this assignment of function was obtained from *in vitro* experiments. Crude extracts of the *gpp* mutants show severely reduced pppGpp hydrolytic

activity. Fractionation of the crude extracts revealed that the *gpp-1* mutation results in the disappearance of two of the five separable pppGpp*ases* which are observed in wild type extracts. The unexpected disappearance of *two* peaks of activity can be accounted for in several ways. One possibility is that the *gpp-1* mutant carries mutations in two closely linked genes of similar function. If this is the case, the genes are not simply tandem repeats of an identical gene, since the products are well separated by DEAE Sephadex chromatography. Verification of this possibility might be accomplished by removing one of the mutations by an appropriate genetic cross. This would result in the recovery of a recombinant with a phenotype which is intermediate between the *gpp-1* mutant and wild type. However, no such recombinants were observed among the several hundred transductants which were scored during the mapping of the *gpp* locus. Thus, if there are two or more mutations, they are either very closely linked or the recombinants cannot be distinguished from the parental phenotypes in mass screening experiments. Since the *gpp* mutants have no readily discerned phenotype, it has not been possible to exclude the possibility of several mutations by the selection of revertants.

A second possibility is that the *gpp*-enzyme is functional as either a monomer and a multimer, or in two different multimeric forms. Since DEAE Sephadex separates on the basis of size and charge, the observed pattern of separation might result. Verification of the multimer hypothesis might be obtained by rechromatography of the individual peak fractions of pppGpp*ase* activity. Spontaneous dissociation of the postulated multimer should result in a shift in the elution profile of one of the *gpp*-specified pppGpp*ases*. A preliminary attempt to demonstrate

the interconversion of the two peaks of *gpp*-enzyme activity revealed that the elution profile of these two activities may vary substantially from one preparation to another (Appendix I). In one instance peak II-A contained the majority of pppGpp*ase* activity, wheras in two other preparations peak II-B was the major peak. Although the reason for this variability is not known, the fact that the two peaks of activity vary inversely suggests that they may be related. These observations are considered as weak evidence in support of the multimer hypothesis.

The substrate specificity of the *gpp*-enzyme was inferred from a series of experiments in which the ability of several other nucleotides to act as competitive inhibitors of the pppGpp*ase* reaction was investigated. The results of these experiments indicated that ATP, GDP, GTP and UTP were not effective inhibitors of the pppGpp*ase* activity. In view of the structural similarity of GTP and pppGpp it appears that the enzyme exhibits a high degree of specificity. Although teleological arguments are seldom compelling, it might be argued that the mere existence of an enzyme which seems to specifically hydrolyze pppGpp to ppGpp is substantial evidence that this pathway is a major route of ppGpp synthesis *in vivo*.

During amino acid starvation the maximal level of pppGpp accumulation in the *gpp* mutants is approximately two-fold greater than the maximal level in the wild type strain. The *in vitro* results suggest that this is due to a reduction in the rate of pppGpp degradation. However, there is no apparent decrease in the rate of pppGpp degradation following the release of starvation conditions. This is presumably due to the presence of several other enzymes which are capable of pppGpp

hydrolysis. Nevertheless, if pppGpp is a major precursor of ppGpp, the increased level of pppGpp accumulation should be accompanied by a proportional decrease in the level of ppGpp accumulation. Since this is not observed, it seems necessary to propose that in the *gpp* mutants, the rate of ppGpp degradation is reduced in proportion to the decreased rate of pppGpp hydrolysis. A simple mechanism to account for this effect is that the *spoT* gene product may not distinguish between ppGpp and pppGpp as substrates. Thus, the increase in the pppGpp pool would reduce the rate of ppGpp hydrolysis by competitive inhibition. Alternatively, it is possible that a *gpp* mutation affects *spoT* function in some other way.

An unlikely alternative to these schemes is that, *in vivo*, the *gpp*-enzyme hydrolyzes pppGpp to something other than ppGpp. This remains a possibility, since it was not rigorously established that the product of the *gpp*-catalyzed reaction was ppGpp. The conditions used for the separation of the reaction products might not have distinguished ppGpp from pppGp. The latter compound would not be expected to be a precursor of ppGpp, since *E. coli* is not known to have an enzyme capable of phosphorylating a 3'-nucleotide.

Several properties of the *gpp* mutants provide convincing evidence that pppGpp is a major precursor of ppGpp. The strongest evidence is derived from the observation that, at the onset of amino acid starvation of *gpp* mutants, the initial rate of ppGpp accumulation lags substantially behind the rate of pppGpp accumulation. This observation is difficult to reconcile with schemes in which ppGpp is the precursor of pppGpp, or in which the two nucleotides are synthesized independently. It is,

however, an essentially qualitative result, in that it does not exclude the possibility that some fraction of the ppGpp pool is synthesized directly from GDP in proportion to the availability of GDP at the site of stringent factor action. However, since all of the enzymes which have been shown to hydrolyze pppGpp reportedly produce ppGpp as the major product, it might be assumed that the amount of ppGpp which is derived from pppGpp is approximately equal to the amount of pppGpp which is hydrolyzed. Since Fiil *et al.* (1970) have calculated that the rate of pppGpp degradation is equal to the rate of ppGpp synthesis, it is concluded that direct synthesis of ppGpp from GDP accounts for a small proportion of the total.

A model for ppGpp and pppGpp synthesis and degradation is presented in Figure 23. The proposed model, which incorporates most of the relevant information, is an elaboration of previous models (Chaloner-Larsson and Yamazaki, 1976; Fiil*et al.*, 1977; Kari *et al.*, 1977). The model proposes that ppGpp and pppGpp are produced by the product of the *relA* gene in response to the binding of an uncharged tRNA to the ribosome. GDP and GTP serve as alternate substrates of the reaction, in proportion to their concentration in the free nucleotide pool. Thus, GTP is the direct precursor of all of the pppGpp produced, wheras GDP is the direct precursor for only some of the ppGpp produced. The pppGpp is subsequently converted to ppGpp by the action of the *gpp*-enzyme, EF-G, EF-T, and possibly other proteins. All or most of the ppGpp is then degraded to an unidentified metabolite by the action of the *spoT* gene product. The probable degradation product of ppGpp is GDP (Sy, 1977). The pathway is regulated in several ways. The synthesis of the purine nucleotides appears to be negatively regulated by the inhibition of

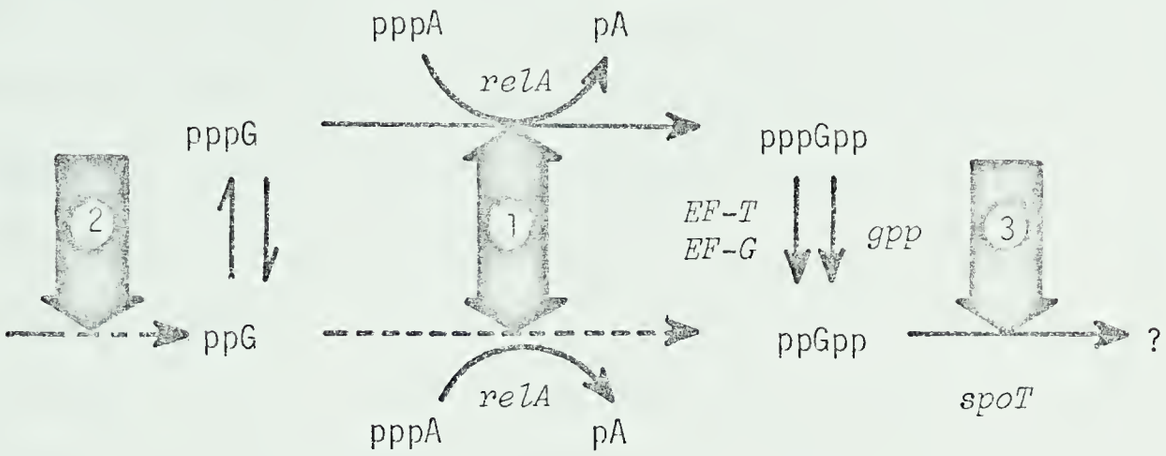

Figure 23 - Proposed model of ppGpp and pppGpp metabolism.
The model proposes that GDP and GTP are converted, respectively, to ppGpp and pppGpp by the *relA* protein in proportion to the free nucleotide pool size of the precursors; pppGpp is converted to ppGpp by the action of the *gpp*-enzyme, EF-G, EF-Tu, and possibly other unidentified proteins; ppGpp is subsequently converted to an unidentified compound by the *spoT* gene product. Possible sites of regulation are indicated by the numbered arrows. The regulatory agents in each case are: (1) the *spoT* gene product, ppGpp, or a metabolite of ppGpp; (2) ppGpp; (3) a component of carbohydrate metabolism.

IMP dehydrogenase and adenylosuccinate synthetase by ppGpp (Gallant *et al.*, 1971). This would reduce the flow of precursors for (p)ppGpp synthesis and would, therefore, be expected to reduce the rate of (p)ppGpp synthesis. The second point of regulation appears to be at the site of (p)ppGpp synthesis. This is inferred from the observation that, during amino acid starvation, *spoT* mutants synthesize (p)ppGpp at lower rates than the wild type. This suggests that either the *spoT* gene product is directly involved in (p)ppGpp synthesis, or that the greater than wild type level of ppGpp accumulation in *spoT* mutants, inhibits (p)ppGpp synthesis. The degradation of ppGpp also appears to be regulated. This is inferred from the observation that, in the wild type strain, the rate of ppGpp degradation is reduced during carbon source downshift (Gallant *et al.*, 1972; Friesen *et al.*, 1975; Hansen *et al.*, 1975). This response suggests that the action of the *spoT* gene product is regulated by some component of carbohydrate or energy metabolism. The model appears to be inadequate to explain the fact that, in *gpp* mutants, the maximal level of ppGpp accumulation is not reduced in proportion to the increase in the pppGpp pool. The resolution of this anomaly requires additional experimental evidence.

Although the search for *gpp* mutants has not resulted in the isolation of mutants which are totally defective in pppGpp hydrolysis, it might facilitate the eventual construction of such a strain. Results presented here indicate that there are at least three other proteins which are capable of pppGpp hydrolysis. It seems probable that the two ribosome-dependent pppGpp*ases* represent EF-G and elongation factor Tu (EF-T), both of which have been reported to possess GTP*ase* and pppGpp*ase* activities (Hamel and Cashel, 1973; Cochran and Byrne, 1974; Block and Haseltine, 1974). Although conditional mutants affecting

these enzymes have been reported (Tocchini-Valentini and Mattoccia, 1968; Lupker *et al.*, 1974), their utility in this context is limited by the fact that they are required for translation, a necessary prerequisite for pppGpp synthesis. Furthermore, the existence of two almost identical copies of the gene for EF-Tu (Jaskunas *et al.*, 1975; Furano, 1977), renders the technical aspects of strain construction difficult. Therefore, it seems that the construction of a multiply mutant strain may not be practical.

In addition to providing evidence that pppGpp is a major precursor of ppGpp, the isolation of the *gpp* mutants may provide an insight into several related problems. The accumulation of pppGpp during downshift of *gpp* mutants was somewhat unexpected since it has been reported that little or no (p)ppGpp synthesis occurs during downshift (Gallant *et al.*, 1972; Friesen *et al.*, 1975; Hansen *et al.*, 1975). The simplest interpretation of the results presented here is that previous authors have underestimated the extent of (p)ppGpp synthesis during downshift, due to the difficulties associated with distinguishing between synthesis and reduced turnover. Alternatively, it is possible that downshift causes a further reduction in the rate of pppGpp degradation in the *gpp* mutants. Inhibition of one or more of the remaining pppGpp*ases* by GDP or a related nucleotide might account for such an effect. Verification of one of these alternatives might result in a useful reappraisal of the mechanism responsible for the downshift induced accumulation of ppGpp.

A particularly interesting and unexpected result was the apparent inviability of *gpp spoT* double mutants. The phenotype expected of such a strain was that it would be partially defective in both ppGpp and pppGpp degradation. Thus, it was expected that the double mutant

would accumulate more pppGpp than a *spoT* mutant, and would have lower levels of ppGpp due to the reduced rate of entry from the pppGpp pool. There is no apparent reason why such a phenotype should be inviable.

The isolation of the presumptive *spoT*^{ts} mutant permitted the construction of a *spoT gpp* strain. Although the *spoT*^{ts} mutant is an atypical *spoT* mutant in several respects, the effect of a *gpp* mutation upon (p)ppGpp accumulation in the *spoT*^{ts} mutant at the non-permissive temperature was as expected. Furthermore, the presence of the *gpp* mutation in the *spoT*^{ts} strain resulted in a dramatic difference in the rate of RNA accumulation at the non-permissive temperature. This result is considered as convincing evidence that the *spoT* and *gpp* genes interact in some manner, but does not necessarily imply that the primary effect of the interaction is at the level of transcription. Since the effect on RNA accumulation was not correlated with the levels of (p)ppGpp, it seems apparent that one or both of these genes are involved in some process other than (p)ppGpp degradation. Further analysis of this problem should be facilitated by a more complete analysis of the *spoT*^{ts} mutant.

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APPENDICES

APPENDIX I

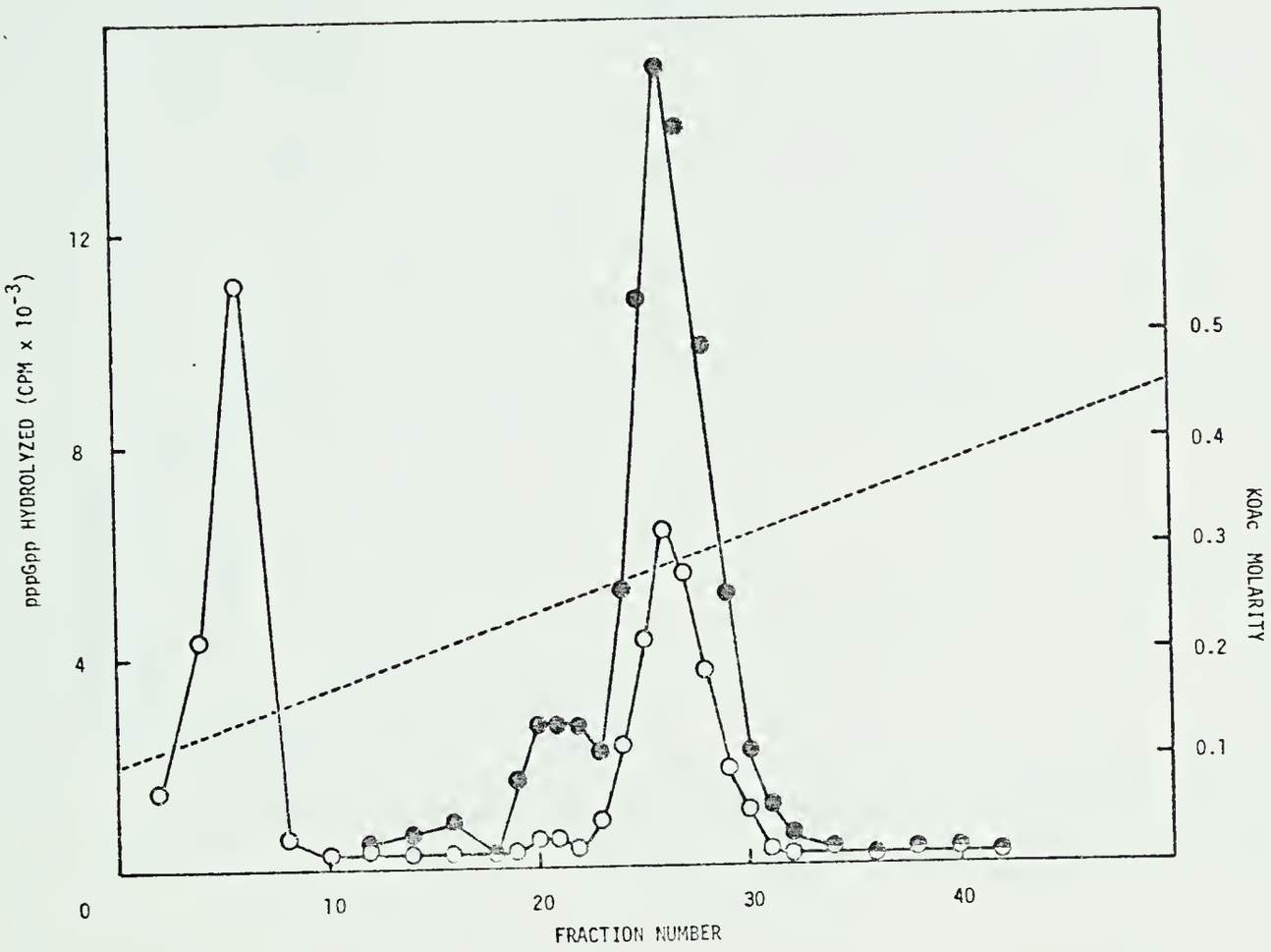

Figure 1 - Ribosome independent pppGpp*ase* activities from the wild type strain. An S-100 extract of the wild type strain CS417 was fractionated on a 1.2 x 33 cm column of DEAE Sephadex. The column was eluted with a 300 ml KOAc gradient (0.1 - 0.45 M) at 10 ml/hr (4°). The elution buffer contained 50 mM Tris-OAc (pH 7.4), 10 mM $Mg(OAc)_2$ and 7 mM 2-mercaptoethanol. The fractions were assayed in a reaction mixture containing 10 ul of pppGpp (10 nmoles), and 25 ul of enzyme. The reaction mixture was incubated at 32° for 60 min. At this time, the reaction was stopped with 5 ul of 5 M formate. The reaction products were separated by chromatography on PEI cellulose, cut out and counted. Symbols (○) pppGpp*ase* activity of undialyzed fractions; (●) pppGpp*ase* activity of fractions which have been dialyzed in buffer-A.

APPENDIX I - continued

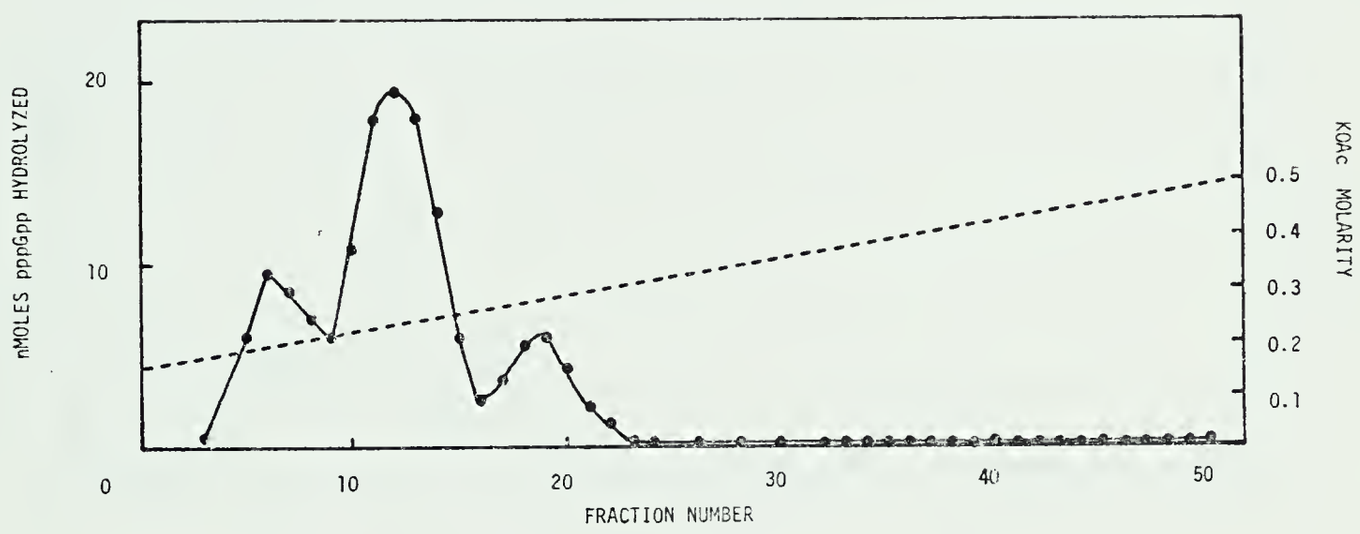

Figure 2 - Ribosome-independent pppGpp*ase* activities in the wild type strain.

An S-100 extract of the wild type strain CS417 was prepared and fractionated as described in Figure 1 except that the KOAc gradient was from 0.15 - 0.5 M. The dialyzed fractions were assayed in 50 ul reactions containing 20 nmoles pppGpp (in 10 ul), 30 ul of protein (in buffer-A), and 10 ul of buffer-A. The reaction was incubated at 32° for 80 min, then terminated by the addition of 5 ul of 5 M formate. A 30 ul aliquot of the reaction mixture was chromatographed on PEI cellulose, and the areas of interest were cut out and counted.

APPENDIX I - continued

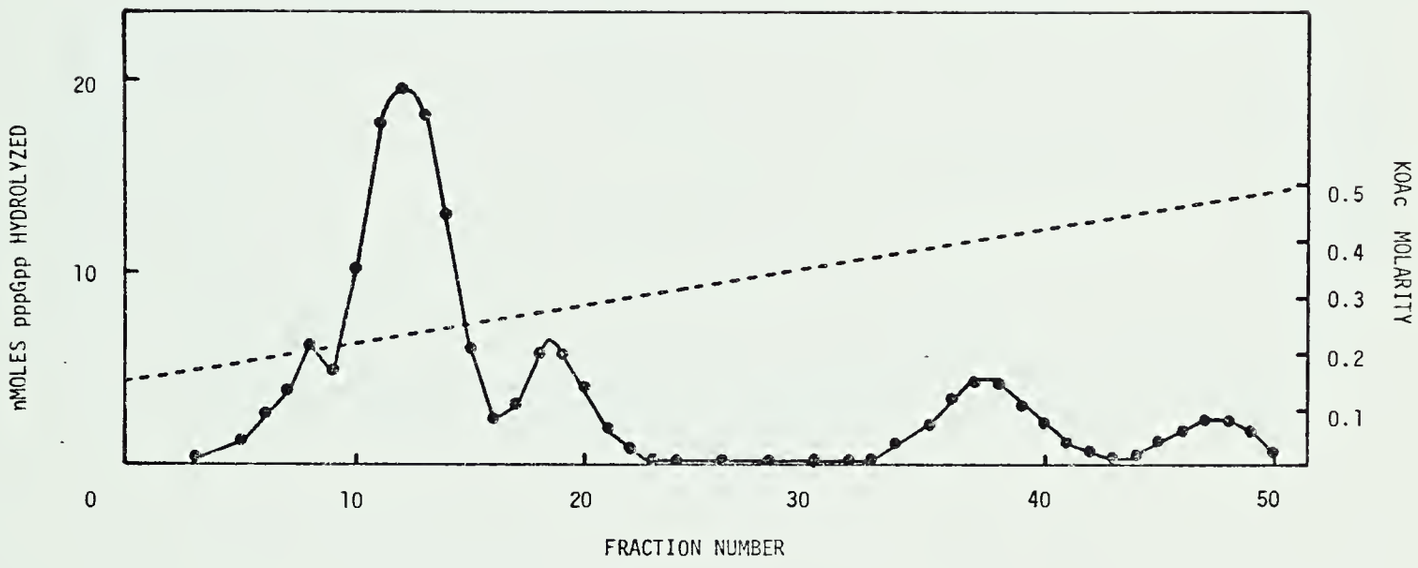

Figure 3 - pppGpp*ase* activities in the wild type strain when assayed in the presence of ribosomes. An S-100 extract of the wild type strain CS417 was prepared, fractionated, and assayed as described in Figure 2 except that 3.0 A_{260} units of low-salt washed ribosomes were included in the reaction mixture.

APPENDIX I - continued

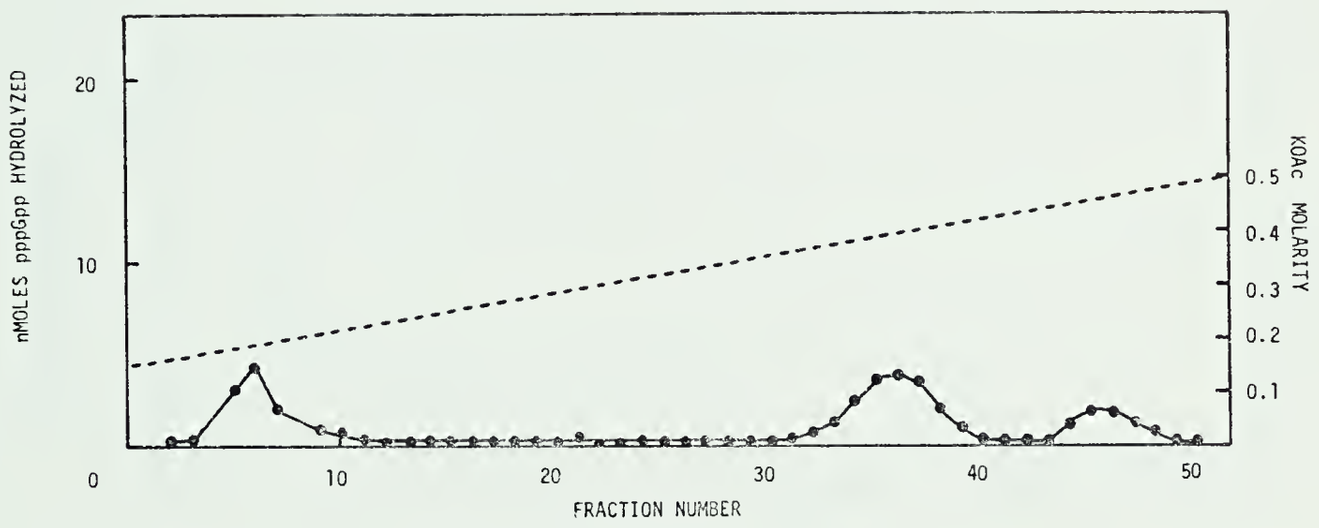

Figure 4 - pppGpp*aee* activities in a *gpp* mutant when assayed in the presence of ribosomes.

An S-100 extract of the *gpp* mutant CS416 was prepared, fractionated, and assayed as described in Figure 3.

APPENDIX I - continued

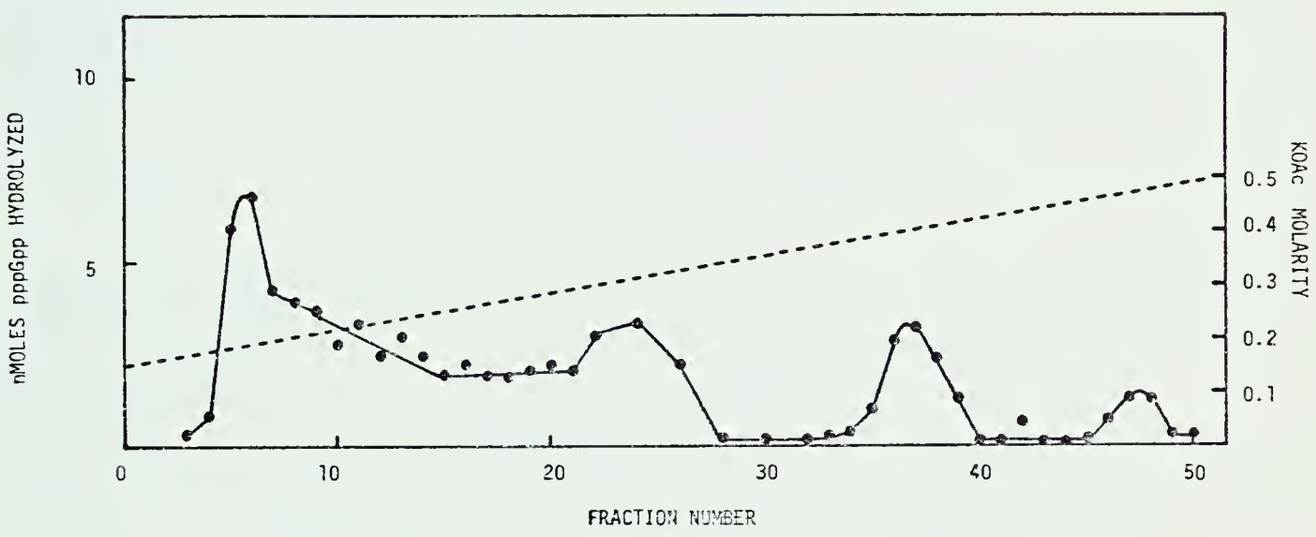

Figure 5 - GTPase activities in the wild type strain when assayed in the presence of ribosomes. An S-100 extract of the wild type strain CS417 was prepared, fractionated, and assayed as described in Figure 3 except that GTP was used as the substrate.

APPENDIX II

Hydrolysis of various nucleotides by 3'-nucleotidase

Substrate	Concentration	% hydrolysis
ppGpp	50 uM	11
pppGpp	80 uM	13
Hydrolysis product of pppGpp*	80 uM	13

The reaction mixture contained substrate, 3'-nucleotidase (1.2 units/ml), and buffer and salts at the same concentration as buffer-A. The reaction was incubated at 32° for 30 min, then terminated by the addition of 0.1 volumes of 5 M formate. The reaction products were separated by chromatography on PEI cellulose thin layers, cut out and counted. The activity of 3'-nucleotidase toward the substrate is presented as the proportion of the substrate which was hydrolyzed during the reaction.

* The hydrolysis product of pppGpp was obtained by incubating the peak fraction of *gpp*-enzyme activity (from a DEAE Sephadex column) with pppGpp until the hydrolysis of pppGpp was complete (2 hrs at 32°). The reaction mixture was then divided into two aliquots, one of which was supplemented with 3'-nucleotidase, and the incubation was continued for an additional 30 min at 32°.